The Impact of Protein-Energy Malnutrition after Stroke on Recovery of Walking in Rats

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By

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ABSTRACT

Understanding comorbidity factors is essential for optimal stroke recovery. Up to 49% of patients develop protein-energy malnutrition (PEM) after stroke, but its effects have not been adequately addressed. The study objectives were to determine the effects of post-stroke PEM on the recovery of skilled locomotion and infarct size.

Adult, male (12-week-old) Sprague-Dawley rats were trained to walk on a regular rung pattern in the horizontal ladder and tested prior to surgery to establish pre-stroke baseline. Rats (now 16 weeks old) were assigned to photothrombotic stroke targeting the forelimb motor cortex or sham surgery. On day 2, rats were tested on the regular ladder rung pattern before assignment to control (12.5% protein) or low protein (0.5%) diets and subdivision into subacute (studied until day 12) or chronic (day 28) groups (n=6-10/group/study). On day 11, rats were tested on the regular pattern and a novel irregular pattern. Chronic study rats were tested again on day 27 on the regular and (no longer novel) irregular patterns. Food intake and body weight were monitored. Serum albumin concentration was measured by a spectrophotometric assay and liver lipid was measured by lipid extraction and dry lipid weight. Infarct volume was measured on cresyl violet stained brain sections.

Feeding the low protein diet resulted in PEM by day 12, based on a decrease in body weight (ANOVA; $p<0.030$), daily food intake ($p<0.019$) and serum albumin concentration ($p<0.001$). A trend for increased liver lipid content in PEM rats became evident on day 28 ($p=0.051$). On the ladder regular pattern, stroke increased error rate only on day 2 (repeated measures ANOVA; $p<0.001$). On the irregular rung pattern, stroke rats had higher error rates relative to shams on days 11 and 27 ($p<0.009$). PEM rats exposed to stroke had more errors than the other groups when the irregular pattern data on d11 were combined from both studies ($p<0.007$). PEM did not alter infarct size on day 12 or 28 (t-test; $p>0.05$).

In conclusion, post-stroke PEM worsened recovery on only the most challenging and novel walking task. Since infarct size was unaltered, other mechanisms are hypothesized to underlie the functional effects.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
µL	Microlitres
µm	Micrometers
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CAP-23	Brain abundant membrane attached signal protein 1
CIMT	Constraint-induced movement therapy
cm	Centimeters
CON	Control diet
CSPGs	Chondroitin sulfate proteoglycans
dL	Decilitres
FOOD	Feed or Ordinary Diet
g	Grams
GAP-43	Growth associated protein-43
hr	Hour
ISCH	Ischemia
kg	Kilograms
L	Litres
M	Molar concentration
MCAO	Middle cerebral artery occlusion
mg	Milligrams
mL	Millilitres
mm	Millimetres
mM	Millimolar
mW	Milliwatts
NFκβ	Nuclear factor kappa beta
nm	Nanometres
PEM	Protein energy malnutrition
PFA	Paraformaldehyde
PT	Photothrombotic
rtPA	Recombinant tissue plasminogen activator
SEM	Standard error of the mean
SHAM	Sham surgery
STAIR	Stroke therapy academic industry roundtable
trkB	Tropomyosin related kinase

Chapter 1 INTRODUCTION

1.1 Rationale

Stroke refers to the sudden loss of brain function due to the disruption of cerebral blood flow. The most common type of stroke is ischemic stroke, which accounts for more than 87% of all stroke incidents (Mozaffarian *et al.*, 2015). Stroke is a significant cause of long-term adult disability in Canada (Lindsay *et al.*, 2010). Upper extremity motor deficit is the most prevalent form among post-stroke disabilities (Kleim *et al.*, 2007). Although limited spontaneous recovery can occur after stroke that can be further promoted by rehabilitation (Murphy & Corbett, 2009), comorbidity factors such as diabetes, hypertension (Ginsberg, 2008), and malnutrition may hinder stroke recovery.

Current stroke treatments are insufficient. Recombinant tissue plasminogen activator (rt-PA), which dissolves the blood clot and restores blood flow, is the only validated medication for treating acute brain ischemia (Casaubon *et al.*, 2015). However, less than 2% of patients receive the treatment (Albers *et al.*, 2011) due to the disadvantage of having to give it within 4.5 hours after stroke and other contraindications (Casaubon *et al.*, 2015). Recently, endovascular thrombectomy has emerged as another useful procedure to decrease mortality rates and improve functional outcome (Goyal *et al.*, 2015). However, the operation requires highly trained health care teams (Meyers *et al.*, 2011) and cannot be used as a universal treatment to stroke.

The past three decades of research focused on discovering neuroprotective agents to disrupt acute ischemic events and to limit the death of injured neurons has had little success (Broussalis *et al.*, 2012). Based on this failure and the emerging science of targeting neuroplasticity as a means of promoting stroke recovery (Carmichael, 2016), alternative strategies have been suggested for the current development of stroke treatments. The first recommendation is to investigate combination therapies to interrupt multiple pathways of the ischemic cascade driving neuronal death, rather than using a single neuroprotectant targeting only one pathway (Savitz & Fisher,

2007; Albers *et al.*, 2011). The second recommendation is to pursue strategies to enhance long-term stroke recovery through brain repair mechanisms, in addition to promoting neuroprotection during the acute phase (Cramer, 2008). Enhanced neuroplasticity plays a critical role in post-stroke brain recovery (Murphy & Corbett, 2009; Overman & Carmichael, 2014). Neuroplasticity refers to the brain reorganization phenomenon characterized by both functional and structural changes in neuronal networks that occurs in response to external experiences, such as practicing musical instruments (Pascual-Leone *et al.*, 2005), playing video games (Bavelier *et al.*, 2010; Hubener & Bonhoeffer, 2014), aerobic exercise (Bavelier *et al.*, 2010), and brain injuries like traumatic brain injury and stroke (Murphy & Corbett, 2009). Post-stroke plasticity is characterized by increased levels of axonal sprouting, dendritogenesis, and synaptogenesis in the peri-infarct region, the region adjacent to the infarct where damaged neurons are still viable (Murphy & Corbett, 2009). Synaptic remodeling activity has been directly correlated with functional outcome after stroke (Carmichael, 2006; Li *et al.*, 2010; Hinman *et al.*, 2013).

The third recommendation is to elucidate the impact of prevalent comorbidity factors, such as diabetes and hypertension, to achieve the optimal outcome after stroke (Ginsberg, 2008). My thesis research focused on investigating effects of post-stroke protein-energy malnutrition (PEM), an under-appreciated comorbidity factor. In industrialized countries such as Canada, protein-energy malnutrition (PEM) is most common in seniors (Chapman, 2006) who are also at highest risk for stroke (Krueger *et al.*, 2015). Anorexia (Soenen & Chapman, 2013), poor dentition, and dementia (Agarwal *et al.*, 2013) are among the many contributors to PEM in elderly individuals. These factors may also account for the 16%-20% of stroke patients that are malnourished at the time of hospital admission for stroke (Yoo *et al.*, 2008). However, the prevalence of PEM increases dramatically after stroke. Within the first week after stroke onset, 20-35% of patients become protein-energy malnourished (Foley *et al.*, 2009); by the time of transferring to a rehabilitation unit, up to 49% are affected by PEM (Finestone *et al.*, 1996; Poels *et al.*, 2006). Dysphagia, or difficulty in swallowing, stands out as the primary risk factor for increased PEM incidence after stroke (Foley *et al.*, 2009). Other factors such as physical impairments also contribute to the elevated post-stroke PEM prevalence (Bouziana & Tziomalos, 2011).

Limited clinical evidence suggests PEM is an independent predictor of poor outcome after stroke. The multicenter FOOD (Feed or Ordinary Food) trial demonstrated a possible relationship between malnutrition and increased functional dependence in the initial cohort of 3012 patients (Food Trial Collaboration, 2003). Evidence from several small clinical studies also suggests that PEM is associated with poor outcome after stroke (Davalos *et al.,* 1996; Martineau *et al.,* 2005; Yoo *et al.*, 2008). However, significant methodological flaws prevent these studies from providing a definite answer to whether PEM is a direct cause of impaired recovery after stroke. Given the limitations of clinical studies to investigate the direct impact of malnutrition on brain repair after stroke, I employed rodent preclinical models in the thesis research to study whether there is a causal relationship between PEM and impaired functional (motor) recovery after cortical stroke.

Using a rat model of cortical stroke, in which the infarct is placed in the forelimb region of the motor cortex, my laboratory colleagues have shown that post-stroke PEM does hinder the recovery of forelimb function. This was evident as decreased spontaneous use of the stroke-affected forelimb during exploration in the cylinder task (Matwee, 2016). However, there are no studies that have investigated the effect of post-stroke PEM on recovery of skilled locomotion. Thus, the goal of the thesis research was to determine whether PEM developing after stroke detrimentally affects the recovery of locomotion, which requires skilled coordination of forelimbs and hindlimbs. I further hypothesized that a detrimental effect of PEM on walking recovery would not be due to increased infarct size, since no such change was found in previous studies from my laboratory, either with PEM pre-existing at stroke onset (Alaverdashvili *et al.*, 2018) or PEM developing after stroke (Matwee, 2016). In addition, it was reasoned that the infarct core would be well developed before PEM would be induced by a low protein diet introduced on day 2 after stroke (Lee *et al.*, 1996). The thesis did not address other mechanisms underlying the effects of post-stroke PEM on skilled locomotion. However, previous findings from my laboratory have demonstrated that brain inflammation, brain plasticity, and the acute phase response may be key contributors to the detrimental effects on recovery from brain ischemia caused by PEM (Ji *et al.*, 2008; Prosser-Loose *et al.*, 2010; Smith *et al.*, 2014; Alaverdashvili *et al.*, 2017).

1.2 Hypothesis

PEM developing after cortical stroke will hinder the recovery of skilled locomotion in adult rats without increasing the infarct size.

1.3 Research Objectives

1) To assess if PEM that develops after cortical stroke hinders the recovery of skilled locomotion in the horizontal ladder walking task.

2) To assess if post-stroke PEM alters the infarct volume.

Chapter 2 LITERATURE REVIEW

2.1 Ischemic Stroke

Stroke refers to the sudden loss of brain function due to the disruption of cerebral blood flow. The most common type of stroke is an ischemic stroke, which is caused by emboli or thrombi occluding cerebral vessels and accounts for more than 87% of all stroke incidents (Mozaffarian *et al.*, 2015). In other cases, the rupture of brain blood vessels induces hemorrhagic stroke (Broussalis *et al.*, 2012). Throughout the thesis, the general term stroke is used to refer to ischemic stroke unless otherwise stated.

Stroke is a significant cause of long-term adult disability in Canada (Lindsay *et al.*, 2010). Recently, it was estimated that approximately 405,000 individuals are living with effects of stroke in Canada (Krueger *et al.*, 2015). It has been projected that by the year 2039 the number of people with permanent stroke disabilities will increase to between 654,000 and 726,000 (Krueger *et al.*, 2015). Depending on the severity and the location of the stroke in the brain, patients can suffer from learning impairment, memory loss, depression, dysphagia and functional motor deficit. Among these post-stroke disabilities, upper limb motor deficit is the most prevalent form (Kleim *et al.*, 2007).

The extent of recovery after stroke is a key factor determining the quality of life and productivity in long-term survivors. Although some limited spontaneous recovery can occur after stroke (Carmichael, 2016) that can be further promoted by rehabilitation (Murphy & Corbett, 2009), comorbidity factors such as diabetes, hypertension (Ginsberg, 2008), and malnutrition may hinder stroke recovery. The study described here focuses on investigating the impact of protein-energy malnutrition (PEM) developing after ischemic stroke, a comorbidity condition affecting up to 49% of stroke patients (Finestone *et al.*, 1996; Poels *et al.*, 2006), on recovery.

2.11 Pathophysiology of Ischemic Stroke

In ischemic stroke, there are sudden loss of specific brain functions due to a disruption of the cerebral blood flow caused by a blood clot (Mozaffarian *et al.*, 2015). Normal cerebral blood flow is approximately 50-60mL/100g brain tissue/min, with less than 10mL/100g brain tissue/min resulting in irreversible neuron death (Broussalis *et al.*, 2012). Two types of blood clots cause ischemic stroke; a local thrombus that is mainly a result of atherosclerosis can cause cerebral artery occlusion, or an embolus can travel into a cerebral artery (mostly in the middle cerebral artery area) and disrupt the blood flow (Broussalis *et al.*, 2012).

The pathophysiology of acute brain ischemia is complex and multifaceted. The initial blood flow reduction rapidly triggers the ischemic cascade, which consists of a series of biochemical reactions that include adenosine triphosphate (ATP) depletion, prolonged depolarization, glutamate excitotoxicity, increased intracellular calcium level, elevated oxidative stress, increased free radical generation, excess activation of pro-inflammatory molecules, and disruption of the blood-brain barrier (Durukan & Tatlisumak, 2007; Broussalis *et al.*, 2012).Within minutes, irreversible neuron death occurs and forms an initial infarct core (Durukan & Tatlisumak, 2007; Heiss, 2012). At the core, most brain cells die through necrosis (Dirnagl *et al.*, 1999). The penumbra refers to the peripheral region adjacent to the infarct core, where blood flow is disrupted to a lesser extent; the ischemic damage is detrimental enough to halt physiological functions of this brain region but not enough to cause immediate neuron death (Paciaroni et al. 2009). Compromised brain cells in the penumbra can proceed to apoptosis (programmed cell death) or may be partially or completely rescued if blood flow is restored promptly. Saving the penumbra is the target for developing acute neuroprotection drug therapy (Broussalis *et al.*, 2012).

2.1.2 Current Acute Stroke Treatments and Neuroprotective Agents

Despite the high prevalence and the devastating effects of stroke, current treatments are insufficient. Recombinant tissue plasminogen activator (t-PA), which dissolves the blood clot and restores blood flow, is the only validated medication for treating acute brain ischemia (Casaubon

et al., 2015). Although international guidelines have extended the administration window of t-PA from within 3 hours to 4.5 hours after stroke, less than 2% of patients receive the treatment (Hacke *et al.*, 2008; Albers *et al.*, 2011). In addition to the large number of patients not arriving at the hospital in time, contraindications disqualify additional patients from receiving this treatment.

Recently, surgical interventions that mechanically remove the blood clot have emerged as another promising approach to treat acute ischemic stroke (Mack, 2016). For instance, the ESCAPE trial has shown that endovascular thrombectomy can decrease mortality rates and improve functional outcome for patients having a small infarct core in a cerebral artery proximal to middle cerebral arteries (Goyal *et al.*, 2015). However, only a small percentage of patients qualify to undergo this surgery, and these procedures require highly trained healthcare teams (Meyers *et al.*, 2011). Therefore, mechanical thrombectomy cannot be used as a universal treatment for stroke.

The research focused on discovering neuroprotective agents to disrupt acute ischemic events and to prevent the death of injured neurons in the penumbra was initiated in the 1980s (Ginsberg, 2008; Broussalis *et al.*, 2012). Despite the many positive findings obtained in animal studies, there has been no successful translation of any neuroprotective agent into the clinic in the past three decades (Ginsberg, 2008; Broussalis *et al.*, 2012). A number of factors account for the failure (Ginsberg, 2008; Broussalis *et al.*, 2012). The primary reason is that in animal studies, neuroprotective agents were often given immediately after stroke or even before stroke whereas such a short therapeutic window is not practical in human patients (Ginsberg, 2008; Broussalis *et al.*, 2012). Moreover, outcome endpoints measured in animal studies are often different from those in clinical trials. Also, young and healthy animals are frequently used whereas stroke patients are commonly elderly and have other comorbidities. Other factors such as the lack of long-term animal studies utilizing functional endpoints (Savitz & Fisher, 2007) and the challenge of studying the diverse stroke types in human patients also contributed to the failed neuroprotection approach (Broussalis *et al.*, 2012).

More efficient restorative interventions are in demand as the number of individuals living with stroke disabilities continues to increase (Krueger *et al.*, 2015). Based on the failure to develop neuroprotection agents and the emerging science of targeting neuroplasticity as a means of promoting long-term stroke recovery (Carmichael, 2016), alternative strategies have been suggested for the current development of stroke therapy. Rather than using a single neuroprotectant targeting only one pathway, therapies aiming to interrupt multiple pathways of the ischemic cascade should be investigated (Savitz & Fisher, 2007; Ginsberg, 2008; Broussalis *et al.*, 2012). In addition to promoting neuroprotection during the acute phase, enhancing long-term stroke recovery through brain repair mechanisms is an alternative approach to treat stroke (Cramer, 2008; Carmichael, 2016). Also, the effects of common comorbidity factors such as diabetes and hypertension on stroke recovery should be understood to achieve optimal recovery (Ginsberg, 2008). My thesis project focuses on understanding protein-energy malnutrition (PEM) developing after stroke, another prevalent comorbidity factor that is under-appreciated.

2.1.3 Post-Stroke Neuroplasticity

Limited spontaneous recovery after stroke can be observed in patients, and this can be further enhanced by interventions such as physical rehabilitation and transcranial magnetic stimulation (Murphy & Corbett, 2009; Corbett *et al.*, 2014). The resolution of some detrimental factors, such as the subsiding of edema, reduced intracranial pressure, and the restoration of cerebral blood flow, account for some of the post-stroke recovery (Lee & van Donkelaar, 1995). Aside from these factors, injury-induced neuroplasticity is another critical mechanism contributing to post-stroke brain recovery (Cramer, 2008; Murphy & Corbett, 2009; Hosp & Luft, 2011; Overman & Carmichael, 2014). In general, neuroplasticity in the brain refers to the brain reorganization phenomenon characterized by both functional and structural changes in neuronal networks (Fu & Zuo, 2011). Previously, it was believed that neuronal networks lost their neuroplasticity by the end of the critical period during early brain development and remained fixed throughout the lifespan (Fu & Zuo, 2011). In recent decades, cumulative data have demonstrated

that in the adult brain, neuronal networks can undergo rewiring and remapping in response to external experiences, such as practicing musical instrument (Pascual-Leone *et al.*, 2005), playing video games (Bavelier *et al.*, 2010; Hubener & Bonhoeffer, 2014), and aerobic exercise (Bavelier *et al.*, 2010).

Plasticity in adult brain can be further stimulated by brain injuries like traumatic brain injury and stroke (Murphy & Corbett, 2009). Similar to developmental plasticity, stroke-induced brain plasticity is characterized by a rapid increase of axonal sprouting, dendritogenesis, and synaptogenesis, which are critical plasticity events contributing to functional recovery (Corbett *et al.*, 2014). Therefore, enhancing post-stroke plasticity has become an essential target to promote optimal stroke recovery. As stated above, upper extremity impairment is the most common deficit observed in human patients, as stroke often causes damage in the sensorimotor cortex (Cassidy & Cramer, 2017). Given this observation, my thesis project focuses on the functional recovery of the forelimb in a rodent photothrombotic stroke model targeting the sensorimotor cortex. The following section outlines some of the current knowledge of post-stroke cortical neuroplasticity.

Structural changes of post-stroke plasticity lead to the remapping and rewiring of certain neuronal networks that can serve as compensation for lost neurons and their contributions to brain functions; this post-stroke reorganization process is considered as an essential mechanism contributing to spontaneous recovery, and it can be further enhanced by external treatments (Brown *et al.*, 2009; Clarkson *et al.*, 2013; Corbett *et al.*, 2015). Structural changes occur in both the peri-infarct region (adjacent to the infarct) as well as distal areas away from the infarct. The majority of changes takes place in the peri-infarct region (Murphy & Corbett, 2009) and are found to positively correlate with functional recovery (Carmichael, 2006; Li *et al.*, 2010; Hinman *et al.*, 2013). In a severe stroke, the contralesional cortex also undergoes reorganization as there is little spared tissue left in the ipsilesional hemisphere (Murphy & Corbett, 2009). However, the role of contralesional cortex in functional recovery remains unclear (Corbett *et al.*, 2015). Furthermore, it has been shown that corticospinal projections descending from the contralesional cortex can cross the midline and make new connections on the denervated side of the spinal cord (Zhao *et al.*, 2013). In addition to the reorganization of existing neuronal networks, neurogenesis (the process

of generating new neurons) has been found to play a limited role in post-stroke functional recovery as well. Upon an ischemic insult, neuron precursor cells originating from the subventricular zone and dentate gyrus in the hippocampus migrate towards the infarct region (Wang *et al.*, 2012). The deprivation of intrinsic neurogenesis has been shown to hinder post-stroke functional recovery, which indicates neurogenesis indeed can contribute to stroke recovery (Wang *et al.*, 2012). In fact, only a few neuron precursor cells reach the peri-infarct region and mature into functional neurons, whereas most of them die during migration (Corbett *et al.*, 2014). These data, and others (Zhao *et al.*, 2008; Bai *et al.*, 2015; Cook *et al.*, 2017), suggest that neuroprotective and neurotrophic factors released from neuron precursor cells, rather than newly born neurons reaching the peri-infarct site and integrating into the network, contribute to the beneficial effect of neurogenesis on functional recovery (Murphy & Corbett, 2009; Corbett *et al.*, 2015).

Heightened structural reorganization in the peri-infarct region occurs along with a series of changes in growth-related genes and molecules that include neurotransmitters, neurotrophins, hormones, and growth factors (Carmichael *et al.*, 2005; Murphy & Corbett, 2009). The majority of changes in the level of these molecules takes place during a critical period when most prominent functional recovery is observed (Wieloch & Nikolich, 2006; Corbett *et al.*, 2015). This critical period can be categorized into four stages based on studies relying on different preclinical stroke models in both rats and mice (Carmichael *et al.*, 2005; Carmichael, 2006; Corbett *et al.*, 2014; Overman & Carmichael, 2014; Corbett *et al.*, 2015): 1) the triggering stage, that occurs in first few days after stroke, when neuroplastic signals are induced by the stroke injury; 2) the initiating stage, at 1 week after stroke, during which levels of growth-promoting molecules are upregulated; 3) the maintenance stage, at 2 weeks after stroke, when extensive axonal sprouting happens; and 4) the maturation stage, at 4 weeks after stroke and beyond, when levels of growth-promoting molecules start to decrease, levels of growth-inhibitory molecules are increased. and new patterns of cortical projections can be detected.

Brain abundant membrane attached signal protein 1 (CAP-23), c-Jun, small proline-rich region protein 1 (SPRR1), and growth-associated protein-43 (GAP-43) are among the most

prominent growth-promoting molecules upregulated in the peri-infarct region (Stroemer *et al.*, 1995; Carmichael *et al.*, 2005). GAP-43 is a protein found abundantly in the growth cone (the tip of a growing axon) during both extensive axonal growth at the early brain development (Denny, 2006) and heightened axonal sprouting in the post-stroke peri-infarct cortex of the adult brain (Carmichael *et al.*, 2005). The level of GAP-43 declines once growth cones collapse and new synapses are formed (Denny, 2006). In the healthy adult brain where limited axonal sprouting takes place, GAP-43 is present at a low level in the primary sensorimotor cortex and other brain regions (Denny, 2006). Both messenger RNA and protein levels of GAP-43 are upregulated in the peri-infarct region within the first few days after stroke, which can last for one month and beyond (Carmichael *et al.*, 2005). It is believed that the upregulation of GAP-43 plays a crucial role in post-stroke axonal sprouting (Carmichael *et al.*, 2005; Carmichael, 2006; Jeffers *et al.*, 2014).

Following axonal sprouting, new synapses are formed between surviving neurons in the peri-infarct region and neurons in the adjacent spared cortex. The elevated level of synaptogenesis results in an increased synaptic density in the peri-infarct region, which is often characterized by an enhanced level of a synaptic protein, synaptophysin. Synaptophysin is the most abundant integral membrane protein (~6-8%) in synaptic vesicles and plays an essential role in neurotransmission (Thiel, 1993). For example, the level of synaptophysin was significantly upregulated after stroke in the middle cerebral artery occlusion (MCAO) model in rats (Stroemer *et al.*, 1992), marking the formation of new connections in the peri-infarct region.

At the maturation stage, levels of growth-promoting molecules start to fall while initially regressed growth-inhibitory molecules begin to upregulate again to facilitate scar formation and to stabilize newly formed connections (Corbett *et al.*, 2014; Overman & Carmichael, 2014). The majority of these inhibitory proteins are myelin-associated proteins such as Nogo A, extracellular matrix proteins such as chondroitin sulfate proteoglycans (CSPGs) and others such as Ephrin-A5 (Carmichael *et al.*, 2005). It has been shown that the deprivation of peri-infarct chondroitin sulfate proteoglycans along with a specific motor training regimen can improve functional outcome in a rat stroke model (Gherardini *et al.*, 2015). Since growth-inhibitory molecules hinder post-stroke

neuroplasticity, blocking their upregulation is considered another treatment approach aimed at extending the growth-promoting time window after stroke.

Ischemic cerebral injury triggers a series of brain inflammatory events that primarily involve microglia, blood-borne macrophages and astrocytes (Anrather & Iadecola, 2016; Kim & Cho, 2016). Once considered as a negative contributor to stroke recovery, a growing body of evidence have demonstrated that the role of brain inflammation in stroke recovery is rather complex as it can be both beneficial and detrimental (Anrather & Iadecola, 2016). Microglia, the primary immune cells resident in the brain, are the first responders to ischemic stroke (Guruswamy & Elali, 2017). Monocyte-derived macrophages, which infiltrate later through the compromised blood-brain-barrier, also play an essential role in neuroinflammation (Kim & Cho, 2016). Depending on the microenvironment, microglia and macrophages can polarize into two primary phenotypes: the M1 subtype with pro-inflammatory nature and the M2 that is anti-inflammatory (Kim & Cho, 2016). Moreover, M1 and M2 subtypes are interchangeable under certain environments (Kim & Cho, 2016). The M1 subtype of microglia and macrophages releases pro-inflammatory cytokines such as tumor necrosis factor α, interleukin 1 and interleukin 6 that lead to increased ischemic injury (Kim & Cho, 2016; Guruswamy & Elali, 2017). The M2 subtype of microglia and macrophages is involved in cleaning up the debris of dead cells (Kim & Cho, 2016; Guruswamy & Elali, 2017). Additionally, M2 subsets secrete anti-inflammatory molecules, including transforming growth factor β and interleukin 10 that dampen inflammation to re-establish tissue homeostasis (Kim & Cho, 2016; Guruswamy & Elali, 2017). Moreover, growth factors, such as vascular endothelial growth factor and insulin growth factor-1, are expressed by microglia and macrophages to facilitate angiogenesis and neuroplasticity processes in the peri-infarct region (Kriz & Lalancette-Hebert, 2009; Iadecola & Anrather, 2011).

Astrocytes become activated upon ischemic insult (Pekny *et al.*, 2014). Reactive astrocytes have several beneficial functions, including removing excessive glutamate (the excitotoxic neurotransmitter released during the ischemic cascade), secreting neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor, and forming a

glial scar that isolates the lesion area from the rest of the brain (Gleichman & Carmichael, 2014; Pekny *et al.*, 2014). The downside of reactive astrocytes is that a range of growth-inhibitory molecules are expressed that would inhibit axonal sprouting (Gleichman & Carmichael, 2014; Pekny *et al.*, 2014). Moreover, astrocytes influence the recruitment and activation of microglia (Pekny *et al.*, 2014). It appears that reactive astrocytes suppress activated microglia while the excess activation of microglia indicates an impaired astrocyte response (Pekny *et al.*, 2014).

In general, ischemic stroke triggers a neuroinflammatory response involving the mobilization and activation of microglia, macrophages, astrocytes, and other peripheral leukocytes. The development of this response greatly impacts the extent of post-stroke neuroplasticity (Gleichman & Carmichael, 2014; Hu *et al.*, 2015) and functional outcome (Iadecola & Anrather, 2011). Furthur understanding of post-stroke neuroinflammation is vital as it may provide therapeutic targets to enhance stroke recovery.

Currently, physical rehabilitation is one of several approaches developed to improve functional recovery by enhancing neuroplasticity. In animal models, enriched rehabilitation is most effective when provided during the early critical period after stroke when neuroplasticity processes are most prominent (Corbett *et al.*, 2015). Enriched rehabilitation can increase synaptic activities in the peri-infarct region and upregulates the level of BDNF (Livingston-Thomas *et al.*, 2016). Another pre-clinical study demonstrated that constraint-induced movement therapy (CIMT) was able to increase the number of synapses from descending corticospinal tracts and to inhibit the level of Nogo A, a growth-inhibitory molecule, in the peri-infarct region (Zhao *et al.*, 2013). Moreover, the combination of a growth factor infusion (epidermal growth factor and erythropoietin) and rehabilitation significantly accelerated functional recovery in animals (Jeffers *et al.*, 2014). Directly blocking CSPGS that are growth-inhibitory also enhanced the effect of a specific training program in rats (Gherardini *et al.*, 2015). However, current clinical rehabilitation regimes are often not sufficient to fully restore impaired functions, and patients are usually left with permanent deficits (Livingston-Thomas *et al.*, 2016). One criticism is that clinical rehabilitation is usually introduced at a late stage rather than during the critical early period

(Corbett *et al.*, 2014). A lack of physical activities and prolonged bed rest within the first few weeks after stroke may further suppress neuroplasticity-induced recovery (Corbett *et al.*, 2014). Therefore, it is now proposed that task-specific physical rehabilitation should be combined with other adjunct therapies to achieve optimal functional recovery. A great effort still needs to be made to fully understand and refine rehabilitation approaches in both human patients and animal models.

Transcranial magnetic stimulation and stem cell therapy are another two restorative treatments under development. In a mouse stroke model, repeated brain stimulations were shown to increase the level of BDNF and nerve growth factor in the peri-infarct region, but no such change was seen in healthy mouse brains, indicating that only injured brains are sensitive to this treatment (Cheng *et al.*, 2014). Transplantation of stem cells into the peri-infarct region has also shown promise to improve functional outcome in animals, but significant disadvantages must be overcome for this to become a reliable treatment (Corbett *et al.*, 2015). Although results from clinical trials of transcranial magnetic stimulation (Klomjai *et al.*, 2015) and stem cell therapy (Eckert *et al.*, 2013) are not definitive, these two approaches still hold great promise to become safe and efficient stroke therapies with further research.

Overall, it is suggested that combinational treatment regimens including physical rehabilitation, socialization, and other adjunct therapies need to be developed for the best recovery (Corbett *et al.*, 2014). Furthermore, understanding and eliminating common co-morbidity factors such as hypertension and diabetes, are essential to achieve optimal functional recovery after stroke (Mozaffarian *et al.*, 2015). My research is based on the premise that another common co-morbidity factor, PEM, could also adversely affect the recovery of walking ability after stroke through affecting the motor cortex.

2.2 Post-Stroke PEM and Recovery from Ischemic Stroke

2.2.1 Prevalence and Causes of PEM in Stroke Patients

Protein-energy malnutrition (PEM) refers to the nutritional status in which protein and energy are inadequate to meet metabolic requirements, resulting in altered body compositions and impaired biological functions (Bouziana & Tziomalos, 2011; van der Pols-Vijlbrief *et al.*, 2014). In industrialized countries such as Canada, PEM is highly prevalent in community-dwelling seniors and nursing home residences (Chapman, 2006; Morley, 2012; van der Pols-Vijlbrief *et al.*, 2014). A German study showed that while 12% of a sample of over 290 seniors receiving home care were malnourished, more than half of the seniors in the study were at risk for malnutrition (Kiesswetter *et al.*, 2013). Seniors are also at the highest risk for developing stroke (Krueger *et al.*, 2015). PEM alone can cause serious adverse effects, including low body weight, decreased muscle mass and strength, increased dependence, and elevated mortality rate (Chapman, 2006; Morley, 2012; Kiesswetter *et al.*, 2013). Anorexia caused by aging and medications, poor dentition, social isolation, and chronic diseases are among contributors to undernutrition that develops in elderly individuals (Chapman, 2006; Bouziana & Tziomalos, 2011; Agarwal *et al.*, 2013; Soenen & Chapman, 2013; van der Pols-Vijlbrief *et al.*, 2014; Sabbouh & Torbey, 2017), and thus may account for many of those cases of PEM that co-exist at the time of stroke. It is estimated that approximately 16%-20% of stroke patients are undernourished at the time of hospital admission for stroke (Finestone *et al.*, 1995; Gariballa *et al.*, 1998; Martineau *et al.*, 2005; Crary *et al.*, 2006; Yoo *et al.*, 2008; Sabbouh & Torbey, 2017).

The prevalence of PEM increases dramatically after stroke (Finestone *et al.*, 1995; Bouziana & Tziomalos, 2011; Peters *et al.*, 2015; Sabbouh & Torbey, 2017). Within the first week after stroke onset, 20-35% of patients become protein-energy malnourished (Foley *et al.*, 2009). By the time of transferring to a rehabilitation unit, up to 49% of stroke patients can be affected by PEM (Finestone *et al.*, 1996; Poels *et al.*, 2006). Differences in assessment time, patient population, and assessing methods can account for the variability in prevalence rates among studies (Marshall, 2016). Dysphagia, or difficulty in swallowing, causes substantially reduced

nutritional intake, and this affects up to 65% of all stroke patients at the acute stage (Gonzalez-Fernandez *et al.*, 2013; Hebert *et al.*, 2016; Sabbouh & Torbey, 2017). Thus, dysphagia stands out as a significant risk factor for malnutrition developing after stroke (Foley *et al.*, 2009; Mould, 2009; Bouziana & Tziomalos, 2011; Cohen *et al.*, 2016). Other factors such as post-stroke depression, dysphasia (impaired speech ability), poor oral hygiene, reduced mobility, and hemiplegia also contribute to impaired self-feeding ability, inadequate nutritional intake, and elevated post-stroke PEM prevalence (Finestone *et al.*, 1995; Bouziana & Tziomalos, 2011; Sabbouh & Torbey, 2017).

Although many nutritional assessment tools, such as Subjective Global Assessment and Mini Nutritional Assessment, have been developed (Morley, 2012; Agarwal *et al.*, 2013; Peters *et al.*, 2015), a gold standard for assessment is lacking for stroke patients. No nutritional assessment protocol has been validated specifically for stroke and its subtypes (Peters *et al.*, 2015). Moreover, invalidated methods or modified, simpler versions were frequently used by registered dietitians for stroke patients when assessed in a recent Canadian national survey conducted across 125 Canadian health institutions (Peters *et al.*, 2015). These drawbacks can lead to the misdiagnosis of PEM and the underestimation of malnutrition rates among stroke patients. Although a persistent issue for the past 20 years, post-stroke malnutrition is under-appreciated and undertreated (Sabbouh & Torbey, 2017). By studying the impact of post-stroke PEM in carefully controlled preclinical models, my research aims to determine the impact of untreated post-stroke PEM on the recovery of walking after cortical stroke. If results are significant, this could enhance clinical awareness of the importance of properly screening and correcting PEM in stroke patients.

2.2.2 Evidence that Post-Stroke PEM Impairs Recovery after Stroke

The FOOD (Feed or Ordinary Food) study, an international multicenter, randomized trial, demonstrated a possible relationship between compromised nutritional status at hospital admission and increased functional dependence at six months after stroke in the initial cohort of 3012 patients (Food Trial Collaboration, 2003). However, significant methodological flaws, such as using

different non-standardized nutritional assessments across centers and the lack of ongoing nutritional monitoring across the study period, prevented this study from providing a definite answer to whether PEM is a direct cause of impaired stroke recovery.

Evidence from several small clinical studies also suggests that PEM is an independent predictor of poor stroke recovery. Pre-existing malnutrition was shown to be associated with an increased length of hospital stay and an increased rate of post-stroke complications (Martineau *et al.*, 2005). Malnutrition developing after stroke is also associated with a higher mortality rate, lower functional scores, and extended hospital stay (Davalos *et al.*, 1996; Sabbouh & Torbey, 2017). Moreover, extensive weight loss and reduced muscle strength result in decreased hand gripping strength, which may reduce the ability of patients to fully engage in rehabilitation (Ha *et al.*, 2010). Another study in ischemic stroke patients showed that undernutrition emerged within the first week of admission independently predicted poor functional outcome at three months (Yoo *et al.*, 2008). Another study found similar results that the worst functional recovery by six months was observed in stroke patients with low body mass indices and low serum albumin levels (Kimura *et al.*, 2017). On the other hand, improved nutritional status during rehabilitation was correlated with better functional outcome (Nishioka *et al.*, 2016). Recently, some clinical studies tried to answer the question whether nutritional supplements can improve functional outcome in undernourished stroke patients, which the large FOOD trial failed to answer (Dennis *et al.*, 2005). For instance, a single-site study found that intensive nutritional treatment given to malnourished stroke patients was correlated with a better motor outcome after rehabilitation (Rabadi *et al.*, 2008). However, this finding was limited by a small sample size and other drawbacks.

Clinical studies have drawn interesting associations between PEM and aspects of post-stroke recovery. However, they have been unable to investigate cause-effect relationships between PEM and motor recovery, brain repair, and its underlying mechanisms after stroke. Thus, our laboratory uses rodent stroke models to investigate whether such a causal relationship exists. Previously, we reported that PEM alone (without stroke) could induce a motor deficit; this was identified as alterations in skilled locomotion in the horizontal ladder walking task in adult rats (Alaverdashvili

et al., 2015a). Recently, we found that PEM pre-existing at stroke onset exacerbated forelimb abnormalities induced by cortical stroke in adult male rats (Alaverdashvili *et al.*, 2018). Also, PEM introduced on day 4 after a photothrombotic stroke (targeted to the forelimb region of the motor cortex) hinders the spontaneous recovery of forelimb function, evident as a decrease in the use of the forelimb affected by the stroke during spontaneous exploration in the cylinder task (Matwee, 2016). In my study, this work is extended to determine whether PEM developing after stroke affects the spontaneous recovery of skilled locomotion, which requires the use and coordination of forelimbs and hindlimbs. This objective was studied using the horizontal ladder task.

2.2.3 Mechanisms by Which Post-Stroke PEM May Impair Recovery

It is proposed that PEM could hinder stroke recovery in several ways. First of all, PEM developing after stroke could impair those mechanisms of post-stroke brain repair (Carmichael, 2016) that are significant players in stroke recovery. Our previous data have demonstrated that pre-existing PEM altered the expression of GAP-43 and tyrosine receptor kinase B (trkB), the primary receptor for BDNF (Prosser-Loose *et al.*, 2010), which might partially explain the poorer functional (cognitive) outcome after global brain ischemia in malnourished gerbils (Bobyn *et al.*, 2005); this model mimics the reduction in blood flow to the entire brain that can occur in conditions such as cardiac arrest (Harukuni & Bhardwaj, 2006). Both GAP-43, an axonal sprouting marker (Carmichael *et al.*, 2017), and BDNF, a neurotrophic factor (Corbett *et al.*, 2015), are essential molecules involved in the events of post-stroke neuroplasticity. However, these findings relate to the pre-existing PEM that is present in 16~20% of stroke patients at admission. My thesis project extends this research to focus on the PEM that develops after stroke, which can affect up to 49% of patients. More recently, our laboratory demonstrated that PEM developing after global brain ischemia decreased the expression of GAP-43 and synaptophysin in the cornu ammonis 3 (CA3) region of the hippocampus at post-stroke day 21 (Smith *et al.*, 2014). These data suggest that brain ischemia-induced plasticity can also be affected by post-stroke PEM. Limitations of this study are that the results are restricted to global ischemia, which primarily affects the cornu ammonis 1

(CA1) subregion of the hippocampus (Harukuni & Bhardwaj, 2006), and the link between the expression of these proteins and functional outcome was not studied. The thesis research addresses the question of whether post-stroke PEM impairs motor recovery after cortical stroke.

Secondly, PEM may alter post-stroke brain inflammation, which, in turn, affects the neuronal repair process. Our previous work has shown that pre-existing PEM increased the reactive gliosis after global ischemia in a subset of gerbils (Bobyn *et al.*, 2005). Further examination demonstrated that pre-existing PEM elevated the activation of hippocampal nuclear factor κB (NFκB), a transcription factor involved in the ischemic cascade during the acute phase after global ischemia (Ji *et al.*, 2008). This suggested that PEM might amplify post-stroke inflammation. However, these findings are limited to global brain ischemia. When pre-existing PEM was examined in a rat cortical stroke model affecting the motor cortex, a mimic of the more common ischemic stroke, reduced astrogliosis and microglia activation were observed in the peri-infarct region (Alaverdashvili *et al.*, 2018). This mechanism may partially explain the elevated stroke-induced walking deficit present in the malnourished rats (Alaverdashvili *et al.*, 2018). It is unknown if this pattern is different when PEM develops after stroke.

Thirdly, PEM can independently induce an acute phase response (Dziedzic, 2015), which could indicate increased systemic inflammation. PEM induced in adult rat results in a decreased concentration of serum albumin, a negative acute phase protein (Andrade Ramos, 2013; Alaverdashvili *et al.*, 2015a). The decrease is also contributed by decreased dietary protein supply. In addition, PEM in adult rats increases the serum concentration of α2-macroglobulin (Alaverdashvili *et al.*, 2015a), a positive acute phase protein (Dziedzic, 2015). While acute phase proteins are nonspecific indicators of systemic inflammation (Gabay & Kushner, 1999), it is noteworthy that poor stroke outcome has been associated with a heightened and/or prolonged acute phase response (Dziedzic, 2008; Idicula *et al.*, 2009) that can be induced by post-stroke PEM.

Lastly, PEM can induce abnormalities in muscle size and function that could detrimentally affect the recovery of walking ability. My laboratory has shown that PEM can cause atrophy of gastrocnemius medialis (Alaverdashvili *et al.*, 2015a), a major muscle in the hindlimb of rats that

contains a high number of fast-twitching muscle fibers relative to slow-twitching muscle fibers (Delp & Duan, 1996). This muscle atrophy can induce muscle weakness and may partially explain the gait abnormality and hindlimb dysfunction observed in malnourished rats (Alaverdashvili *et al.*, 2015a). While muscle wasting and weakness caused by protein-energy malnutrition or protein deficiency is a well-acknowledged phenomenon in clinical populations (Norman *et al.*, 2011), further study is required to determine to what extent PEM can hinder stroke recovery by inducing skeletal muscle dysfunction.

2.3 Stroke Models of Focal Ischemia in Rodents

2.3.1 Overview

Given the highly heterogeneous nature of human stroke, many stroke models in rodents aim to address different aspects of the complexity in clinical stroke (Harukuni & Bhardwaj, 2006; Corbett *et al.*, 2017; McCabe *et al.*, 2017; Sommer, 2017). Researchers should choose a model based on their study objectives and consideration of strengths and limitations of the model. There are two main categories of preclinical stroke models: global ischemia models, such as the two-vessel occlusion model, induce a reduction in blood flow to the entire brain (Harukuni & Bhardwaj, 2006), and focal ischemia models result in a localized infarct core in a specific region of the brain (Kumar *et al.*, 2016).

Among focal ischemia models available, most commonly used models are middle cerebral artery occlusion (MCAO) model, endothelin-1 vasoconstriction model and photothrombotic model (Corbett *et al.*, 2017; McCabe *et al.*, 2017). To overcome the previous failure of translational research solely relying on young and healthy animals, both sexes, older animals, and animals with various clinically relevant comorbidity factors should be used as they better resemble the majority of clinical patients (Fisher *et al.*, 2009; Corbett *et al.*, 2017; McCabe *et al.*, 2017; Sommer, 2017). Thus, studying the comorbidity factor, PEM, addresses a critical translational issue for promoting recovery after stroke.

Several methods are used to induce MCAO. Distal MCAO can be induced by transiently clipping distal branches of the middle cerebral artery whereas permanent occlusion can be achieved by cauterizing its branches (Durukan & Tatlisumak, 2007; Corbett *et al.*, 2017). This method requires highly-trained surgeons and invasive surgical procedures, including craniotomy (Durukan & Tatlisumak, 2007; Murphy & Corbett, 2009; Fluri *et al.*, 2015). The infarct produced by distal MCAO is primarily confined to the cortex (Durukan & Tatlisumak, 2007; Murphy & Corbett, 2009). Alternatively, a more significant injury can be produced by occluding proximal ends of the middle cerebral artery (Murphy & Corbett, 2009). The occlusion can also be achieved by inserting an intraluminal filament (or a coated suture) through internal cerebral artery into the origin of the middle cerebral artery and the filament can be withdrawn or left in to achieve transient or permanent occlusion (Kumar *et al.*, 2016; McCabe *et al.*, 2017; Sommer, 2017). Proximal MCAO produces a massive infarct affecting both the striatum and the cortex, which is commonly seen in clinical stroke (Corbett *et al.*, 2017; McCabe *et al.*, 2017). Although various types of filament have been designed to overcome the problem (McCabe *et al.*, 2017), this method still carries a high risk of subarachnoid hemorrhage, and the infarct size is inconsistent (Fluri *et al.*, 2015; Kumar *et al.*, 2016). Another variation of MCAO is to introduce an artificial embolus (a blood clot) into the internal carotid artery (Bacigaluppi *et al.*, 2010; Kumar *et al.*, 2016; McCabe *et al.*, 2017; Sommer, 2017). If the embolus breaks down, multiple infarcts can be generated (McCabe *et al.*, 2017). This model provides an excellent tool to study thrombolytic therapies and is the closest to mimicking a human ischemic stroke (Kumar *et al.*, 2016; McCabe *et al.*, 2017). However, the high mortality rate limits this model from being frequently used (Murphy & Corbett, 2009). Overall, advantages of the MCAO model are that the location of the infarct closely mimics the majority of clinical strokes as they often occur in the region of middle cerebral arteries and the model creates a well-defined penumbra of considerable volume, with mechanisms of repair at play that are very valuable for studying neuronal repair (Lo, 2008; Corbett *et al.*, 2017; Sommer, 2017). However, due to the involvement of invasive surgical procedures and the low reproducibility, MCAO models are not ideal for my study.

Local injection of endothelin-1, a potent vasoconstrictor, can produce ischemic stroke by constricting blood vessels (Durukan & Tatlisumak, 2007; Murphy & Corbett, 2009; Corbett *et al.*, 2017; McCabe *et al.*, 2017). Endothelin-1 can be stereotaxically injected into a region of the brain parenchyma to produce a cortical stroke or be injected near a middle cerebral artery to achieve a damage involving subcortical structures such as the striatum (Corbett *et al.*, 2017; McCabe *et al.*, 2017). Although the method is relatively easy, many disadvantages have been reported. Firstly, the infarct does not occur at the injection site, therefore it can be difficult to be precise (Bacigaluppi *et al.*, 2010; Kumar *et al.*, 2016). Secondly, endothelin-1, independent of its ability to cause vasoconstriction, can alter the activities of astrocytes and axonal sprouting, which may interfere with interpreting effects of treatment or a co-morbidity factor on recovery (Durukan & Tatlisumak, 2007; Kumar *et al.*, 2016). Hence, this model is not a good choice for my study.

2.3.2 Photothrombotic Stroke Model

The photothrombotic stroke model chosen for my study has several advantages for addressing my research questions. This model has proved useful in studying motor function (Moon *et al.*, 2009; Becker *et al.*, 2016) and mechanisms of neuronal repair and restoration (Carmichael *et al.*, 2005; Clarkson *et al.*, 2013). It was initially developed by Watson and his colleagues in 1985 (Watson *et al.*, 1985). In this model, an infarct is induced through the transcranial activation of a photosensitive dye, Rose Bengal, with a laser beam that has a wavelength of 532nm. The activation, in turn, generates reactive oxygen species, triggers the coagulation cascade, and forms a permanent thrombosis (Watson *et al.*, 1985; McCabe *et al.*, 2017). Our laboratory has recently published a refined method where the laser intensity can be tightly controlled and carefully monitored to increase the consistency of infarct size (Alaverdashvili *et al.*, 2018).

This technique has many advantages, such as high reproducibility, less invasive surgery (e.g., no craniotomy), and a relatively simple technique (Corbett *et al.*, 2017). Being less surgically invasive is the primary consideration for my thesis project as invasive surgical procedures can

induce a hypermetabolic response that causes marked nutritional depletion, tissue wasting, and elevated energy and protein requirements (Twyman, 1997; Chapple *et al.*, 2016); this is not a feature of stroke patients (Finestone *et al.*, 2003). Therefore, the photothrombotic model was chosen for my thesis project to best mimic the nutritional and metabolic status of the clinical stroke population.

In addition, the infarct can be induced in any specific location in the cortex, and the size can be accurately defined with stereotaxis. The photothrombotic model also has limitations. Firstly, the peri-infarct region, where the majority neuronal repair activities occur, is limited in size. In addition, it produces an immediate and vast vasogenic edema that is not seen in human stroke (Corbett *et al.*, 2017). Furthermore, the rapidly developed photothrombosis is unusual in human stroke (Durukan & Tatlisumak, 2007; Bacigaluppi *et al.*, 2010; McCabe *et al.*, 2017). Although this makes it unsuitable for studying neuroprotective treatments, this is not a goal of my study. It is an excellent choice to explore stroke-induced motor deficits and neuroplasticity (Carmichael *et al.*, 2005; Moon *et al.*, 2009; Clarkson *et al.*, 2013) as such cellular changes in its peri-infarct region are comparable to those in the penumbra of MCAO models (Lee *et al.*, 1996; Corbett *et al.*, 2017).

2.4 Functional Assessment in Focal Ischemia Stroke Models in Rats

2.4.1 Overview

The Stroke Therapy Academic Industry Roundtable reports, initially developed in 1999 (Stroke Therapy Academic Industry, 1999) and later updated in 2009 (Fisher *et al.*, 2009) have emphasized the importance of measuring not only the infarct size (volume) but also functional outcomes in animal models of stroke; functional recovery is the primary endpoint in clinical trials, and histological findings do not always correlate well with the functional response in humans (Stroke Therapy Academic Industry, 1999). Both reports also point out that outcomes should be assessed during both the acute phase and the long term, which allows a dynamic picture of the

functional response and to establish whether any findings are sustained (Stroke Therapy Academic Industry, 1999; Fisher *et al.*, 2009). Many behavior tests have been developed to evaluate post-stroke motor function in rats. These include cylinder test (Schallert *et al.*, 1997), Montoya staircase (Montoya *et al.*, 1991), single pellet reaching (Whishaw *et al.*, 1991), beam walking task (Goldstein & Davis, 1990), and horizontal ladder walking task (Metz & Whishaw, 2002). In my study, horizontal ladder walking task was selected to assess the walking ability in rats.

2.4.2 Horizontal Ladder Walking Task

The horizontal ladder walking task is a sensitive test that can quantitatively and qualitatively measure the skilled locomotion of each limb as well as the coordination between limbs (Metz & Whishaw, 2002; 2009). Side walls of the ladder are made of transparent Plexiglass, which are 19 cm high and 1m long (Metz & Whishaw, 2002). Metal rungs (3mm diameter) are inserted into holes spaced evenly at 1mm on the bottom of the walls to create a walking platform. A basic, regular rung pattern is formed when rungs are placed into every other hole; this pattern is used in both training and functional assessment. Moreover, rungs can be spaced unevenly to create more challenging patterns that prevent rats from learning both relative and absolute locations of the rungs and thus increase the sensitivity of the test (Metz & Whishaw, 2002; 2009). A recent study demonstrated that the sensitivity could be further increased by changing the inclination of the ladder (upward or downward) (Antonow-Schlorke *et al.*, 2013).

After training, rats traverse the ladder back and forth without any reinforcement. The walking process of each rat on the ladder is videotaped and the foot placement of a limb in every step is scored frame-by-frame using a 7-category scale (Metz & Whishaw, 2002; 2009). Different scores are assigned to 7 type of limb placements, which include: *correct placement* when the mid-portion of a paw is placed on the rung (score of "6"), *partial placement* when a limb is placed using either wrist/heel or digits/toes (score of "5"), *correction* when a limb is aimed for one rung but is placed on another or when a limb is quickly repositioned on one rung (score of "4"),

replacement when a limb is quickly shifted to another rung after being placed on the first rung (score of "3"), *slight slip* when a limb slips off a rung but does not cause a fall (score of "2"), *deep slip* when a limb slips off a rung and causes a fall (score of "1"), and *total miss* when a limb completely misses a rung and cause a fall (score of "0") . An error refers to either a total miss, a deep slip, or a slight slip (Metz & Whishaw, 2002; 2009). The number of each type of foot placement and errors are counted and frequencies of each type of foot placement and errors relative to total steps are used as behaviour endpoints.

The horizontal ladder walking task has been validated as a valuable behavioral test to assess skilled locomotion deficits in the motor system induced by stroke (Metz & Whishaw, 2009; Schonfeld *et al.*, 2017). Moreover, it has been widely used in a body of pre-clinical stroke studies to evaluate the efficacy of drug therapies and rehabilitation regimen (Emerick & Kartje, 2004; Ploughman *et al.*, 2007; Garcia-Alias *et al.*, 2009; Paquette *et al.*, 2009; Beltran *et al.*, 2010; Sun *et al.*, 2013; Jiang *et al.*, 2016). For instance, using the ladder rung walking task one study demonstrated that rehabilitation initiated early after stroke significantly improved functional outcome but rehabilitation started 30 days after stroke had no such effect (Biernaskie *et al.*, 2004). In another study, early anti-inflammatory intervention combined with rehabilitation improved functional recovery, as detected by a dramatically decreased error rate and an increased correct placement frequency of the stroke-affected forelimb in the ladder rung walking task (Liebigt *et al.*, 2012).

Many advantages are associated with the use of the horizontal ladder walking task. The training process and each training session is relatively quick compared to other complex tests such as Montoya staircase and single pellet reaching (Schonfeld *et al.*, 2017). The task allows a comprehensive examination of spontaneous walking ability in trained animals (Metz & Whishaw, 2009). The ability to vary rung spacing provides the opportunity to further increase the sensitivity by challenging animals to walk on an unfamiliar surface (Schonfeld *et al.*, 2017). More importantly for nutrition studies, food restriction is not required during testing (Metz & Whishaw, 2009), which is crucial in nutritional studies to avoid confounding the closely controlled nutritional intake. A

disadvantage, as with other behaviour tests that use ordinal rating scales, the interval between two scores from the 7-category scale in ladder rung walking does not reflect the numerical difference between two types of placements (Muir & Webb, 2000). Another limitation of this test is that scoring the walking ability frame-by-frame is greatly time consuming (Balkaya *et al.*, 2017).

Chapter 3 METHODS AND MATERIALS

All experiments were approved by the University of Saskatchewan Animal Research Ethics Board and adhere to the Canadian Council on Animal Care guidelines for humane animal use.

3.1 Experimental Design

Male, adult (12 weeks old) Sprague-Dawley (SD) rats (n = 87) were obtained from Charles River (QC, Canada). Rats were housed in pairs in a temperature (22^{o}C) and humidity-controlled suite with a 12-hour light/dark cycle (7AM to 7PM). Food and water were provided *ad libitum* throughout the study, except for a short period of 5 days of food restriction (80% of *ad libitum* daily food intake) introduced at the beginning of the horizontal ladder task training.

The experimental design is shown in Figure **3.1**. After acclimatizing for 1 week, rats were handled (5 min/rat) for 3 days and were then trained for 11 days on the horizontal ladder walking task. All rat started to receive the purified control diet (12.5% protein) 1 week before surgeries. Each rat was tested on the horizontal ladder on the 4th day prior to surgery to obtain pre-stroke baseline data. On day 0, each animal was assigned to either a photothrombotic stroke or a sham surgery. To assess functional deficits during the acute phase after stroke, each rat was tested in the horizontal ladder on day 2, after which rats were assigned to receive either the control diet or a low protein diet (0.5% protein).

Hence, two independent variables, nutritional intervention (CON or PEM) and surgical treatment (SHAM or ISCH), yielded four subgroups: i) the group of rats that received control diet and underwent sham surgery (CON-SHAM, n=22); ii) the group of rats that received control diet and underwent stroke surgery (CON-ISCH, n=20); iii) the group of rats that received the low protein diet and underwent sham surgery (PEM-SHAM, n=25); iv) the group of rats that received the low protein diet and underwent stroke surgery (PEM-ISCH, n=20). Animals from these 4 subgroups were then assigned to either the subacute study (euthanized at post-stroke day 12, CON-

SHAM n=8, CON-ISCH n=10, PEM-SHAM n=9, PEM-ISCH n=10) or the chronic study (euthanized at post-stroke day 28, CON-SHAM n=14, CON-ISCH n=10, PEM-SHAM n=16, PEM-ISCH n=10). In the subacute study, each rat was tested again in the horizontal ladder on post-stroke day 11. In the chronic study, additional testing on the ladder was done on post-stroke days 11 and 27. At the time of euthanasia and after perfusion, serum, liver, and brain samples were collected and stored at -80^{o}C for further analysis.

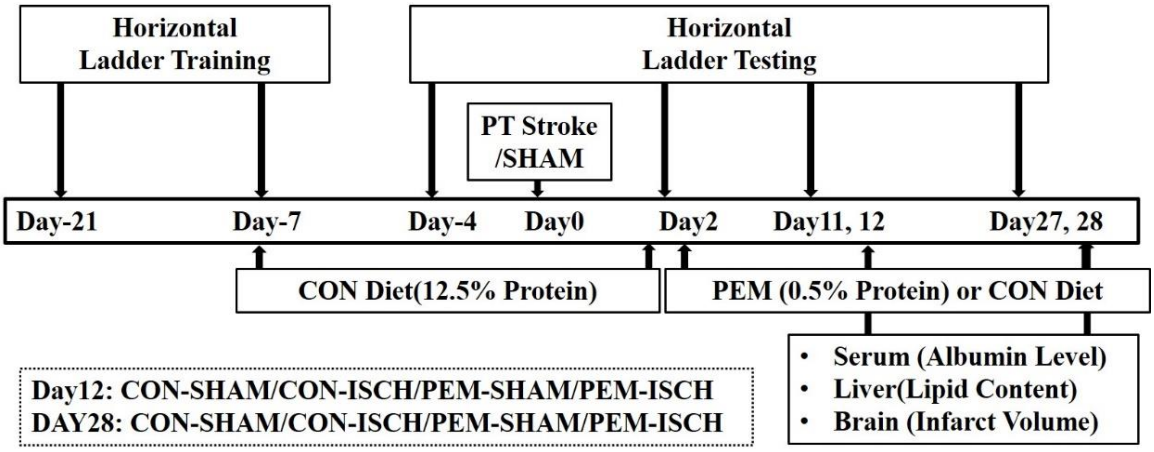

Figure 3.1 Experimental design. PT = photothrombotic.

3.2 Horizontal Ladder Walking Training and Functional Assessment

Skilled walking was assessed through the horizontal ladder walking task. The method was adapted from the original version (Metz & Whishaw, 2002).

The horizontal ladder apparatus is composed of a transparent box [121cm x 15cm x 41cm (W x D x H)] with 4 clear Plexiglass side walls and an open bottom and top (**Figure 3.2**). To form a walking platform, metal rungs (3mm diameter) are inserted into holes (1cm apart) at the bottom of two side walls. A mirror is placed underneath the platform to facilitate visualizing the movements of the rats. The difficulty of the test can be altered by changing distances between rungs. In addition, employing different rung patterns prevents rats from memorizing absolute and

relative locations of rungs and hence increases the sensitivity of this test (Metz & Whishaw, 2002). At the training stage and pre-stroke baseline and acute phase testing, only the regular pattern (**Figure 3.3A**) was used, in which all rungs are 2cm apart. On days 11 and 27, animals were tested on both the regular pattern as well as an irregular pattern, in which distances between rungs alternate between 2cm and 3cm (**Figure 3.3B**).

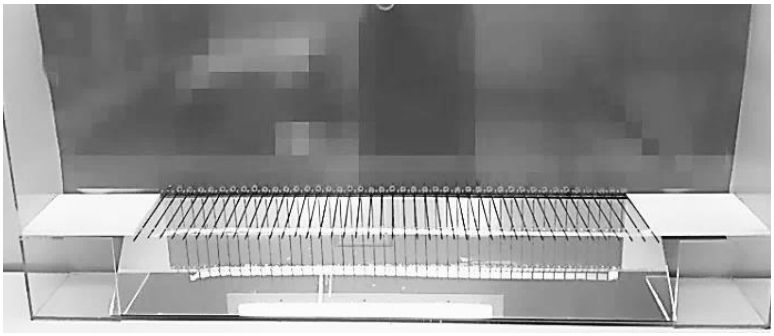

Figure 3.2 Horizontal ladder apparatus shown with the regular rung pattern.

Figure 3.3 Illustrative images of the regular rung pattern (A) and the irregular rung pattern (B).

3.2.1 Training

Each animal received one training session per day, and each session typically lasted 20-40 minutes depending on the activity level of the animal. On day 1, rats were handled in the colony room (5min/rat). On day 2, banana-flavored sugar pellets (Bio-Serv #F05986, 10 pellets/rat) were given into cages after handling in the colony room. By doing so, rats became familiar with the food reward that would be used to encourage ladder walking during the training. On day 3, rats were handled and given sugar pellets in the behaviour suite. During the next 5 days (days 4-8), the rats were food restricted to 80% of their daily *ad libitum* food intake recorded from days 1 to 3; this was to motivate animals to walk across the ladder to obtain sugar pellets placed on the opposite

end. Once eaten, pellets were replaced immediately to maintain the motivation. During days 4 to 6, two cage-mates were placed on the same ladder and could cross the ladder back and forth. Individual rat walking was initiated on day 7 and was continued until the end of the training. On day 9, free access to *ad libitum* food in their home cages was resumed and continued until the end of the experiment. On days 12 and 13, video recording equipment (a Sony 3CCD camcorder under a cold light source (Lowel Satellite 4)) was set up during the training to acclimatize rats to avoid the possibility of neophobia on testing days. By day 14, each individual rat was able to walk across the ladder without long pauses or hesitations, which indicated successful training (Metz & Whishaw, 2002).

3.2.2 Pre-Stroke Baseline Testing

To measure the pre-stroke baseline performance, animals were tested on the regular rung pattern on the 4th day prior to surgeries. For each rat, 5 ladder crossings were recorded by the camcorder with a shutter speed of $1/1000s^{-1}$. One crossing was considered as a rat walking from one end of the ladder to the other.

3.3 Photothrombotic Stroke and Sham Surgery

The procedure was modified from the original model (Watson *et al.*, 1985) as described in a recent publication from our laboratory (Alaverdashvili *et al.*, 2015b). The infarct was placed in the forelimb region of the motor cortex of the left hemisphere to induce a functional deficit in the contralateral (right) forelimb of the rat.

The rats were now approximately 16 weeks old (112-116 days). Anaesthesia induction was with 5% isoflurane and 100% O_2. The scalp between the eyes and ears was shaved, and the tail was cleaned using warm water and chlorhexidine soap. The animal was then mounted facing down on a stereotaxic frame and was kept anaesthetized under a mixture of 2-4% isoflurane in

60%N_2O/40%O_2. Throughout the surgery, physiological parameters including pulse, oxygen saturation and respiration rate, were continuously monitored using a MouseOx Plus, a multiparameter monitor (Starr Life Science). To maintain the body temperature at approximately 37^oC, a feedback-regulated homoeothermic blanket was placed underneath the rat throughout the surgery; the temperature probe of the blanket was lubricated with Vaseline and inserted into the anus of the animal. A syringe containing rose Bengal (10mg/kg, prepared on the same day of the surgery and covered with aluminium foil to avoid photodegradation) was placed between the blanket and the animal to maintain a physiological temperature.

The shaved area on the head was cleaned 3 times with chlorhexidine soap followed by 3 times with 70% ethanol. The remaining procedures followed aseptic techniques. A 2-3cm incision was made from the eyes to the ears along the midline. Coordinates of a 4x4 mm^2 square on the skull (+3mm to -1mm anterior-posterior from Bregma and +1mm to +5mm lateral of midline) were defined by the stereotaxic system using a surgical bone drill. The skull was thinned with the drill directly over this area until underlying blood vessels were visualized. During the thinning process, drops of sterile saline and sterile cotton tips were used to clean the debris. A light-proof metal mask with a 4x4 mm^2 window cut-out was centered on the thinned region of the skull to ensure a precise illumination to the region of interest to increase the reproducibility of the infarct. To aid visualizing tail veins, the tail was warmed up in warm water (~40^oC) before a catheter (BD Insyte, 24GA 0.75IN) was inserted into a lateral tail vein. In some cases, a tourniquet was applied to the proximal end of the tail to help visualize tail veins. Once the catheter was inserted successfully and was filled with blood, the pre-warmed 1mL syringe (Luer Lock) filled with rose Bengal was attached to the catheter. The solution was slowly injected over 30 seconds following by flushing with ~0.5mL sterile saline. Upon finishing the injection, the pre-warmed laser beam (532nm, Beta Electronic, 4mm diameter) with a power density of approximately 290mW/cm^2, which was controlled by a variable neutral density (ND) filter (Alaverdashvili *et al.*, 2015b), was immediately placed over the thinned area for 10 minutes to induce ischemia. For sham surgeries, the rose Bengal was injected but no laser irradiation was applied. Following the laser illumination,

the incision was cleaned with sterile saline and sutured up (Look Nylon Suture, 4-0 USP). A dose of bupivacaine (2mg/kg, Marcaine®) was injected subcutaneously at the base of the incision site, and sterile saline (10mg/kg/hr) was administered subcutaneously in the nape of the neck to replace fluid losses.

The rat was then placed on a clean paper towel in a clean cage. The cage was positioned such that only half the cage was on a warm heating blanket; thus, the animal, once mobile, had the option to choose the cool or warm side during recovery. Water was provided immediately after the procedure, and food was returned into the cage after one hour. Rats were closely monitored for the next 4 days, and after that, they were checked a minimum of once per day until the end of the study. Any signs of discomfort or tail inflammation were reported to the supervisor, and counsel was sought with the veterinarian. The need for euthanasia prior to the end of the study was rare but was performed according to our laboratory human intervention point checklist.

3.4 Post-Stroke Diet Assignment

The composition of the control diet and the low protein diet purchased from Dyets, Inc. (PA, USA) was modified from the American Institute of Nutrition (AIN)-93M diet (Reeves *et al.*, 1993), as shown in **Table 3.1**. The antioxidant, tertiary-butylhydroquinone, included in the AIN-93M diet was excluded from the purified diets. The control diet (12.5% protein) and the low protein diet (0.5% protein) are calorically equivalent. Our laboratory has previously shown that 16 week old male SD rats fed with the low-protein diet (0.5% protein) voluntarily decreased their food intake and thus reduced their caloric intake, which consequently produced a mixed state of protein and energy malnutrition (Andrade Ramos, 2013). Food intake per cage was recorded daily, and the body weight of each rat was recorded twice a week, except during the initial 4 days after surgery when the body weight was measured daily.

Table 3.1 Composition of experimental diets[a]

Components	**Control Diet (g/kg) (12.5% Protein)**	**Low Protein Diet (g/kg) (0.5% Protein)**
Vitamin Free Casein	140.0	5.7
L-Cystine[b]	1.8	0.1
Sucrose	100.0	100.0
Cornstarch	465.7	556.8
Dextrinized Cornstarch	155.0	184.0
Soybean Oil (without tBHQ)	40.0	40.0
Cellulose	50.0	50.0
Mineral Mix[c]	35.0	0
Mineral Mix[d]	0	35.0
Calcium Phosphate, dibasic	0	13.0
Calcium Carbonate	0	2.9
Vitamin Mix[e]	10.0	10.0
Choline Bitartrate	2.5	2.5

[a]*Purified diets were purchased from Dyets Inc. (Bethlehem, PA, USA).*
[b]*The amount of L-cystine was proportional to the quantity of casein.*
[c]*AIN-93M mineral mix (Reeves et al., 1993).*
[d]*Modified AIN-93M mineral mix with the deletion of calcium and phosphorus, an increase of potassium citrate·H_2O from 28.0 to 226.6g/kg, and an increase of sucrose from 209.8 to 618.3g/kg.*
[e]*AIN-93M vitamin mix (Reeves et al., 1993).*

3.5 Post-Stroke Functional Testing

There were three post-surgical testing times on the horizontal ladder: i) post-surgical day 2 on the regular rung pattern, ii) post-surgical day 11 on both the regular and the irregular rung patterns; iii) post-surgical day 27 on both the regular and the same irregular patterns (only for the chronic study). For each rung pattern on each testing day, 5 ladder crossings were recorded for each individual rat. Walking performance on the regular pattern was tested prior to that on the irregular pattern. Between the two testing sessions on the same day, animals were returned to their colony room to rest for at least 1 hour to avoid low activity in the ladder on the second test.

3.5.1 Analysis of Skilled Walking

Recorded videos were analyzed frame-by-frame (Quick Time Software) by our laboratory technician, Megan Morgan, who was blinded to experimental groups. Based on the original method (Metz & Whishaw, 2002), the frequency of the rat making an error with its stroke-targeted forelimb was tabulated. An error refers to a slight slip, a deep slip or a total miss (Metz & Whishaw, 2002). A 'slight slip' is when the targeted forelimb slips off a rung but does not cause a fall, whereas a 'deep slip' refers to when the targeted forelimb slips off a rung and causes a fall. A 'total miss' is when the targeted forelimb falls between the rungs without contacting any rungs and causes a fall. The total number of errors relative to the total number of steps walked yields the frequency of foot fault errors.

3.6 Tissue Collection

On post-surgical day 12 (subacute study) or 28 (chronic study), animals were deeply anesthetized under a mixture of 5% isoflurane and 100% O_2. Before perfusion, approximately 3mL blood was taken directly from the left ventricle of the heart and was collected into a glass tube to obtain the serum (see below). The animal was then transcardially perfused with saline for 5 minutes (~20mL/min). After saline perfusion, one lobe of the liver was removed and was immediately flash-frozen in liquid nitrogen. The perfusion was continued using 4% paraformaldehyde (PFA) solution for another 5 minutes (~20mL/min). The animal was then decapitated, and the brain was carefully extracted from the skull and preserved in ~200mL 4% PFA solution at first 24 hours. Upon PFA preservation, the brain was sequentially immersed into a series of sucrose solutions with increasing concentrations (10% to 20% to 30% sucrose in 0.1M phosphate buffer) to achieve cryoprotection and to avoid freezing artifacts. Once the brain sample sank to the bottom of the container in the final 30% sucrose solution, the cerebellum was removed. The rest of the brain sample was embedded in the optimal cutting temperature (OCT) compound (Tissue-Tek) and was frozen in liquid isopentane bathed in a mixture of dry-ice/acetone (-78^{o}C) for 5 minutes or until no

bubbles were formed. All samples (blood serum, the liver, and the brain) were then stored at -80^{o}C until further analysis.

3.7 Serum Albumin Concentration

After being collected into a glass tube, the blood was left to clot at room temperature for 45 to 90 minutes before being centrifuged at 1,500 x g (Allegra 25R Centrifuge, Beckman Coulter) for 10 minutes at 4^{o}C. The serum was then aliquoted into several 0.6mL Eppendorf tubes chilled on ice before being stored at -80^{o}C.

The serum albumin concentration was determined by the bromocresol green method (Dumas et al. 1997). A series of standard solutions (2, 3, 4, 5, 6, and 7g/dL), made up from a stock bovine serum albumin solution (10g/dL), were used to plot the standard curve. A volume of 25µL of a standard, blank, or a serum sample was added to 5.0mL working bromocresol green solution (3 parts 0.1M succinate buffer, 1 part 0.6mM bromocresol green solution, and 4 g/L of 30% Brij-35 [pH 4.2]). The blank contained only 5.025mL working bromocresol green solution. Each mixture was mixed 5 times using a micropipette to ensure homogeneity. After sitting for 30 minutes at room temperature, 1mL of each mixture was pipetted into a 1.5mL disposable cuvette. The absorbance of each cuvette was read at 628 nm (SpectraMax M5, Molecular Devices). Blanks, standard solutions and serum samples were run in triplicate. A linear regression curve of absorbance was plotted relative to concentrations of standard solutions after subtracting the absorbance of blank samples. The concentration of each sample (in triplicate) was obtained from the linear regression analysis. The mean of the three concentrations was recorded as the concentration of the serum sample.

3.8 Total Liver Lipid Content

Liver lipid content was also analyzed as an indicator of protein-energy status, since it is increased in clinical PEM (Badaloo *et al.*, 2005). Frozen liver samples were moved from -80^{o}C to -20^{o}C storage one day prior to the analysis. On the next day, the samples were thawed at room temperature for 1 hour. Approximately 0.5g of each liver sample was cut, weighed and placed into a pre-labelled glass vacutainer tubes. Next, 1 mL of 0.15M sodium chloride solution was added to each tube. The mixture in the tube was then homogenized (Polytron) at the speed of setting 7 for approximately 30 seconds. Another 1mL of 0.15M sodium chloride solution was used to rinse the homogenizer blade and was collected into the tube. After adding 5mL of a 2:1 mixture of chloroform and methanol, the tube was capped with a rubber stopper before being centrifuged at 2,175 x g (Beckman Centrifuge Allegra 25 with TS-5.1 rotor) for 10 minutes at room temperature. Next, the upper aqueous layer was discarded, and approximately 1 gram of anhydrous sodium sulfate was added to the remaining mixture. After vigorously shaking the mixture, the solid was filtered out (#1 Whatman filter paper) and the filtrate was collected into another pre-weighed glass tube. The chloroform was evaporated using a vacuum centrifuge (30^{o}C, 100millibars) for 2 hours. Tubes were cooled down to the room temperature before being re-weighed. The weight of dry liver lipid was obtained by subtracting the weight of the test tube from the weight after evaporation (a combined weight of test tube and final sample). Percent liver lipid content was determined as follows: the weight of the dry lipid/the wet weight of the liver sample × 100%. The analysis was run in duplicate (two specimens from the same liver), and the mean was recorded as the total liver lipid content.

3.9 Assessment of Infarct Volume

To assess the infarct volume in the brains collected from ischemic groups, frozen brains were sectioned into 28μm thick coronal slices that extended throughout the region of the infarct. Sham brains were also sectioned at the same brain coordinates to determine whether there was any unexpected cortical damage. Serial sections at 420μm intervals were stained with cresyl violet and then scanned into JPEG images (20x magnification, ScanScope CS scanner by Aperio).

Due to some staining and processing artifacts, the infarct volume was estimated using two different methods. In the first method, the infarct area in the cortex of each section was directly outlined and the size was measured using the software, ImageJ (the National Institutes of Health, https://imagej.nih.gov/ij/). The infarct volume was calculated using the following formula:

Infarct volume in the cortex of the injured hemisphere *= average area of the infarct in the cortex of the injured hemisphere x interval between sections x number of sections.*

Although the intent was to place the infarct in the motor cortex, there was damage in the underlying corpus callosum in some brains. Ischemia in the white matter triggers different cell death pathways and neuronal repair mechanisms from those in the grey matter (Sozmen *et al.*, 2009). Therefore, it is important to distinguish the damage in the cortex from that in the corpus callosum (See Appendix A). The damaged area in the corpus callosum was traced in each section and was measured using ImageJ. The volume of brain damage in the corpus callosum and the total infarct volume were calculated using following formulas:

Infarct volume in the corpus callosum *= average area of the infarct in the corpus callosum in the injured hemisphere x interval between sections x number of sections.*

Total infarct volume in the injured hemisphere *= infarct volume in the cortex + infarct volume in the corpus callosum*

In the second method, areas of healthy uninjured cortex in each hemisphere were outlined and measured through ImageJ software. The region defined as cortical tissue was determined by using anatomical hallmarks (Paxinos & Watson, 2007). Although it is ideal to include the entire

hemisphere in such assessments (Clark *et al.*, 2008), only the cortex in each hemisphere was measured due to staining and sectioning artifacts that damaged subcortical structures and precluded the possibility of outlining the entire hemisphere. The tissue loss in the injured hemisphere was calculated using following formulas modified from (Clark *et al.*, 2008):

Volume of tissue loss *= remaining volume of the healthy uninjured cortex in the normal hemisphere – remaining volume of the healthy uninjured cortex in the injured hemisphere.*

Remaining healthy uninjured cortex in a hemisphere *= average area of the remaining cortex in a hemisphere x interval between sections x number of sections.*

When sections were missing, data were calculated by summing areas in each available section and multiplying by the distance from the first and the last section acquired.

To assess whether there was any unexpected cortical damage that occurred during sham surgeries, stained sections of sham brains were carefully examined under a microscope (Nikon, H550L). Damage to the cortex in a sham brain was visualized as a cluster of dark, brown stained nuclei that represented necrotic tissue, comparing to purple stained healthy cells.

3.10 Statistical Analysis

Statistical analyses were performed using SPSS 24.0 for Windows. For all analyses, a significant difference was considered as $p<0.05$. Data are described as mean ± SEM.

To determine whether there was any inherent physiological bias prior to the diet assignment on post-stroke day 2, physiological parameters (oxygen saturation, pulse rate, and the body temperature) acquired during surgeries for both subacute and chronic studies were analyzed using 2-factorial Analysis of Variance (ANOVA).

Body weight and food intake data collected prior to the diet assignment on post-surgical day 2 were evaluated through independent t-test, with surgery as the independent variable. Body

weight data collected after the diet assignment were analyzed through 3-factorial repeated ANOVA, with surgery and diet as between-subject variables and time as the within-subject variable. An independent t-test was used to determine when changes in body weight first became significant between the pooled diet groups. Sample size was insufficient to conduct repeated measures ANOVA on the food intake data, since this was collected on a cage basis (with 1-2 rats per cage). Thus, daily food intake after diet assignment was analyzed by 2-factorial ANOVA until a significant diet effect emerged. The serum albumin concentration and liver lipid content were analyzed by 2-factorial ANOVA.

The error rate on the regular rung pattern of the horizontal ladder walking task was analyzed by 3-factorial repeated ANOVA, with time as the within-subject variable and diet and surgery as between-subject variables. The focus was to analyze data collected from the chronic study (pre-stroke baseline and post-stroke days 2, 11, and 27). However, the data from the subacute study (pre-stroke baseline and post-stroke days 2 and 11) were also analyzed by the same approach to determine if results were consistent with those from the chronic study. As step 1, 3-factorial repeated ANOVA was performed on the error rate from pre-stroke baseline and post-stroke day 2 to determine: a) whether there was any inherent bias in pre-stroke ladder training prior to assignment to diet and surgery groups (the independent variables), b) if stroke induced a functional deficit, and c) if there was any inherent bias in surgical groups prior to the dietary assignment on post-stroke day 2. When there was a significant time x surgery interaction, an independent t-test was performed on the pooled ISCH and SHAM groups at each time point to determine whether there was a significant stroke effect. As step 2, 3-factorial repeated ANOVA was performed on data from days 2, 11, and 27 to determine whether diet (PEM or CON) affected the error rate of the targeted forelimb.

For the analysis of data from the irregular rung pattern, a repeated ANOVA was not performed on the chronic study data (collected on days 11 and 27) since the irregular rung pattern was only novel on day 11 but not on day 27. Instead, the data were analyzed separately on each day by 2-factorial ANOVA, with diet and surgery as independent variables. Because the irregular

rung pattern was tested on day 11 in both subacute and chronic studies, the day 11 data from the two studies were combined and analyzed by 2-factorial ANOVA to examine how the results would be influenced by an increased sample size. When there was a diet x surgery interaction, a *post-hoc* Tukey's HSD test was performed.

An independent t-test was used to compare infarct volume between CON-ISCH and PEM-ISCH groups for the subacute and chronic studies.

Chapter 4 RESULTS

4.1 Excluded Animals

Prior to the completion of the experiment, two animals were excluded. One CON-ISCH rat from the chronic study was excluded due to an inflamed tail from multiple failed attempts to inject the Rose Bengal dye in the tail vein during the surgical procedure. The other (CON-SHAM rat from the chronic study) was excluded due to an unexplained hyperplastic growth on the proximal region of the tail, which could induce systemic inflammation and potentially confound the experimental results. Upon a histological (cresyl violet staining) examination of the serial brain sections and the review of surgical records, one CON-ISCH rat from the subacute study was excluded since unsuccessful tail vein injections failed to induce brain ischemia. In addition, 22 rats from sham groups (8 - CON-SHAM - chronic study; 10 - PEM-SHAM - chronic study; 2 - CON-SHAM – subacute; 2 - PEM-SHAM – subacute) were excluded due to unintended damage to cortical layers I and II in the surgically manipulated hemisphere. This was attributed to excessive force applied during drilling to thin the skull during surgeries.

4.2 Physiological Parameters from Surgeries

Physiological parameters, including the level of oxygen saturation, pulse rate and the body temperature, measured during surgeries are shown in **Table 4.1 A & B**, respectively. The data were compared among the 4 assigned groups to assess whether there was any inherent physiological bias prior to the diet assignment on day 2.

In both studies, levels of oxygen saturation were well maintained across 4 groups. Two-factorial ANOVA revealed that there was no surgical effect ($p_{subacute}$=0.243, $p_{chronic}$=0.121), diet effect ($p_{subacute}$=0.066, $p_{chronic}$=0.617), or surgery x diet interaction ($p_{subacute}$=0.115, $p_{chronic}$=0.345) on mean oxygen saturation. There was no surgery effect ($p_{subacute}$=0.736, $p_{chronic}$=0.496), diet effect ($p_{subacute}$=0.806, $p_{chronic}$=0.869), or surgery x diet interaction ($p_{subacute}$=0.432, $p_{chronic}$=0.567) on

mean pulse rate in either study. Ischemia significantly increased the body temperature in both the subacute ($p_{subacute}$=0.007) and chronic ($p_{chronic}$=0.001) studies. There was no diet effect ($p_{subacute}$=0.056, $p_{chronic}$=0.586) or surgery x diet interaction ($p_{subacute}$=0.814, $p_{chronic}$=0.892). The mean body temperature of combined ischemic groups (CON-ISCH and PEM-ISCH) was significantly higher than that of combined sham groups (CON-SHAM and PEM-SHAM).

Table 4.1 Physiological parameters measured during surgeries

A) Subacute study

Experimental Group ‡	Oxygen Saturation^ (%)	Pulse Rate^ (per min)	Body Temperature^ (^oC)
CON-SHAM	99 ± 0.1	321 ± 7.3	36.8 ± 0.2
CON-ISCH	99 ± 0.1	325 ± 8.5	37.2 ± 0.2 *
PEM-SHAM	98 ± 0.6	331 ± 13.2	36.4 ± 0.2
PEM-ISCH	99 ± 0.1	320 ± 8.8	36.9 ± 0.1 *

*^ Physiological parameters are presented as mean ± SEM. ‡ CON-SHAM, n=6; CON-ISCH, n=9; PEM-SHAM, n=7; PEM-ISCH, n=10. * Body temperature of ISCH groups was significantly higher than that of SHAM groups (2-factor ANOVA, p=0.007)*

B) Chronic study

Experimental Group ‡	Oxygen Saturation^ (%)	Pulse Rate^ (per min)	Body Temperature^ (^oC)
CON-SHAM	99 ± 0.1	330 ± 13.3	36.7 ± 0.1
CON-ISCH	99 ± 0.2	328 ± 12.6	37.3 ± 0.1 *
PEM-SHAM	99 ± 0.2	337 ± 7.2	36.7 ± 0.2
PEM-ISCH	99 ± 0.2	324 ± 4.4	37.4 ± 0.2 *

*^ Physiological parameters are presented as mean ± SEM. ‡ CON-SHAM, n=6; CON-ISCH, n=10; PEM-SHAM, n=6; PEM-ISCH, n=10. * Body temperature of ISCH groups was significantly higher than that of SHAM groups (2-factor ANOVA, p=0.001)*

4.3 Body Weight

Post-surgical body weights over the 28-day period in the chronic study are shown in **Figure 4.1**. Prior to the diet assignment on day 2, body weights between pooled SHAM and ISCH groups were not different from each other (independent t-test, p=0.074). Three-factorial repeated

ANOVA analysis of body weights after day 2 demonstrated a significant time x diet effect (within-subject effect, p=0.001). Neither a significant time x surgery effect (p=0.999) nor a significant time x surgery x diet effect (p=0.996) was revealed. An independent t-test demonstrated that the body weight of the pooled PEM groups first became significantly lower than that of pooled CON groups on day 15 (p=0.008) and there was a trend on day 11 (p=0.059). Over the 28 days, the two CON groups increased their body weight from the baseline (the day of surgery) by a mean of 17.7% whereas the two PEM groups decreased theirs by 8.6%. This resulted in a difference of 22.0% between control and malnourished animals in the final body weight on day 28.

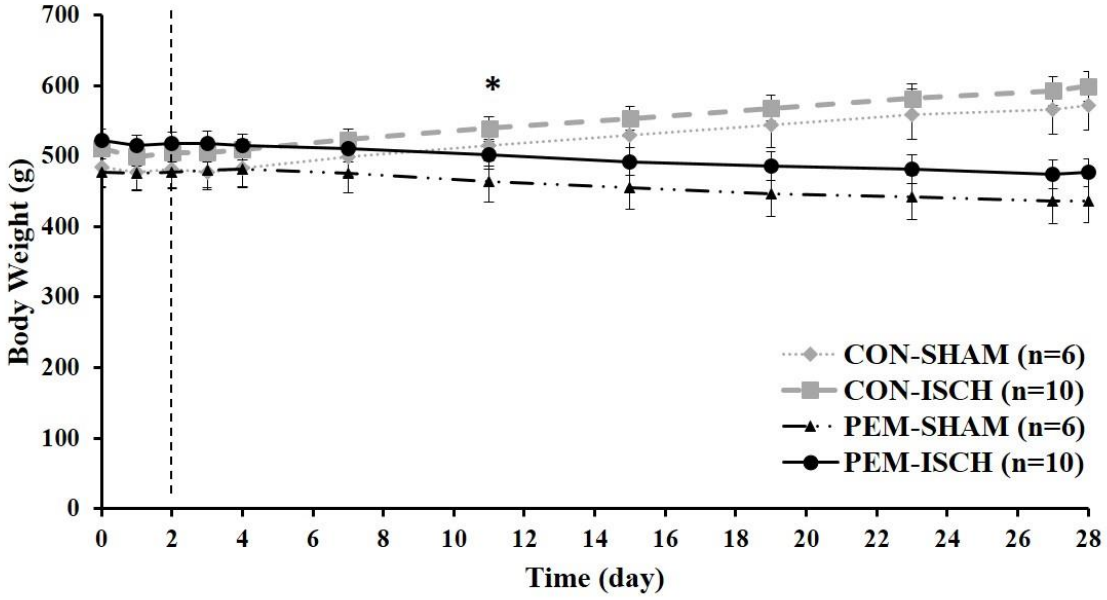

Figure 4.1 Mean (±SEM) body weight in the chronic study. Day 0 was the surgery day (the baseline). Dashed vertical line indicates the day on which rats were assigned to receive either control or low protein diet. * indicates the first day on which the body weight of the combined PEM groups became significantly decreased (independent t-test, p=0.008).

Body weights from the subacute study are shown in **Figure 4.2.** Note the higher body weights at the baseline, even though they were received at an identical age to those rats in the chronic study. A bias was detected on day 0 in that the rats to be assigned to SHAM surgery weighed significantly more that those to be assigned to ISCH surgery (independent t-test; p=0.038). This bias of higher body weight in the pooled SHAM groups persisted to day 2 after surgery and prior to the diet assignment (independent t-test, p=0.028). Nonetheless, the body weight change in the subacute study (**Figure 4.2**) showed a similar pattern to that of the first 12 days in the chronic study (**Figure 4.1**). That is, the low protein diet fed groups (PEM) gradually lost weight whereas control diet fed rats continuously gained weight. Three-factorial repeated measures ANOVA applied to body weights from days 3 to 12 revealed a significant time x diet effect (within-subject effect, p<0.001). The lower body weight in the PEM groups first became significant on day 11 (p=0.030). By day 12, CON animals had increased their body weight by 5.5%, compared to their baseline body weight. PEM rats decreased their body weight by 3.3 % over the 12 days. This resulted in a difference of 12.9% in the final body weight between control and malnourished animals on day 12.

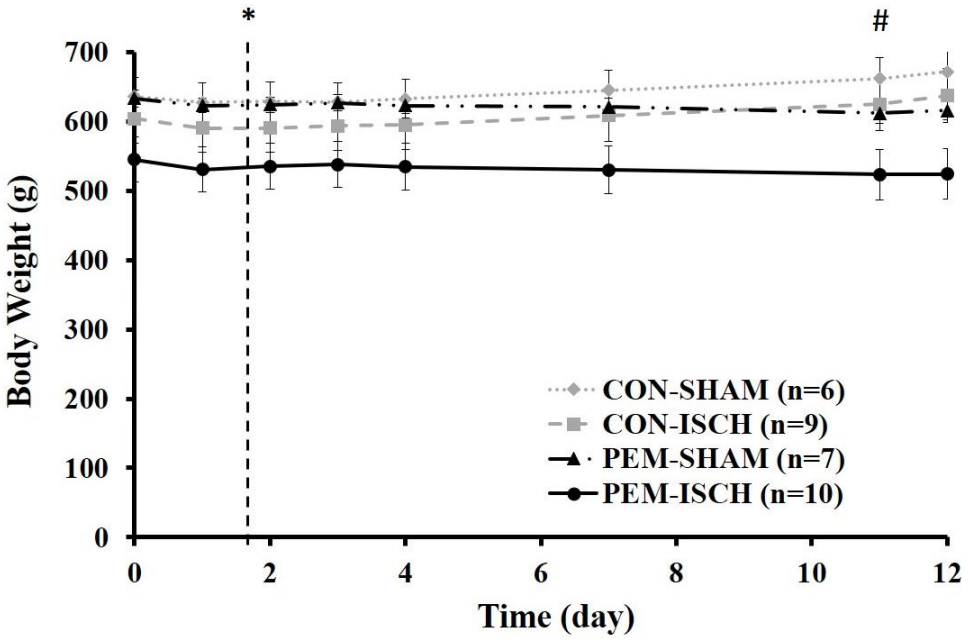

Figure 4.2 Mean (±SEM) body weight in the subacute study. Day 0 was the surgery day (the baseline). The dashed vertical line indicates the day on which rats were assigned to receive either control or low protein diet. *Indicates an inherent bias at baseline in surgical group, with pooled SHAM groups weighing more than ISCH groups (independent t-test, p=0.038). #Indicates the first day on which the pooled PEM groups weighed significantly less than the pooled CON groups (independent t-test, p=0.030).

4.4 Food Intake

The daily food intake per rat collected on a cage basis from the chronic study is shown in **Figure 4.3**. Prior to the diet assignment, the food intake of pooled SHAM and ISCH groups was not significantly different (independent t-test, p=0.453). Two-factorial ANOVA analysis applied to daily food intake after the diet assignment identified an independent diet effect beginning on post-surgical day 11, with the PEM groups eating significantly less (p=0.015). Neither an independent surgical effect nor a surgery x diet interaction was found at any time point (p>0.05). The difference in average food intake between control and PEM groups during the period between day 3 and day 28 was 19.7%.

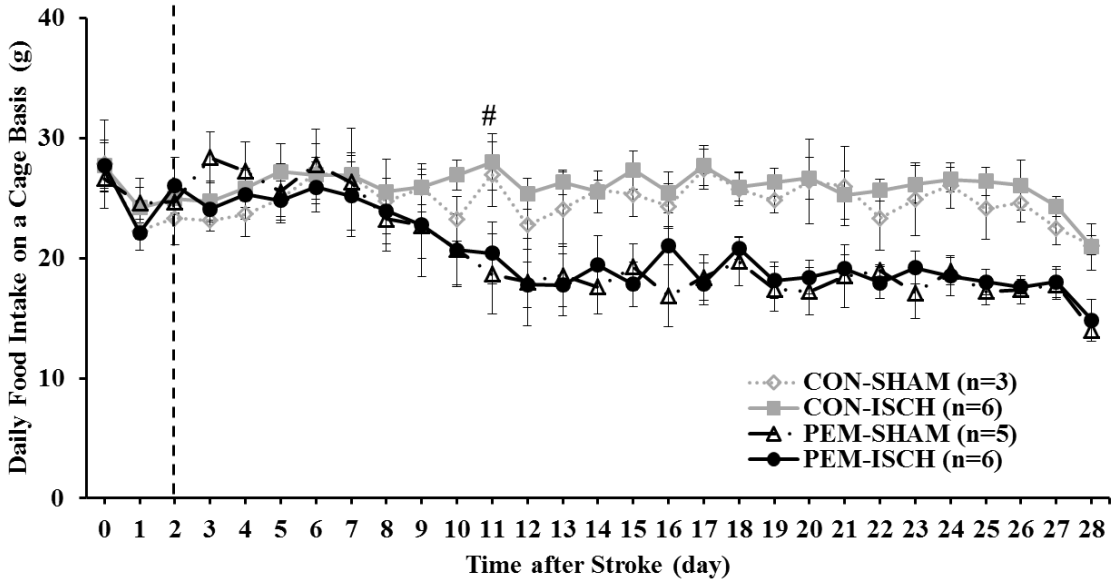

Figure 4.3 Mean (±SEM) daily food intake (g/rat) in the chronic study estimated based on cage data. Day 0 was the surgery day (the baseline). The dashed vertical line indicates the day on which rats were assigned to receive either control or low protein diet. # indicates the first day on which the food intake of pooled PEM groups first became significantly lower than that of CON groups (2-factor ANOVA; p=0.015).

The daily food intake per rat in the subacute study is shown in **Figure 4.4**. On day 1, the food intake of pooled ISCH groups was significantly lower than that of pooled SHAM groups (independent t-test, p=0.022). However, no difference was found between SHAM and ISCH groups on either day 0 or day 2 prior to diet assignment (independent t-test, p_{day0}=0.123, p_{day2}=0.156). After day 2 (diet assignment), the pooled PEM groups gradually reduced food intake, which became significantly lower than that of the CON groups on day 12 (2-factor ANOVA, p=0.025). Neither an independent surgical effect nor a surgery x diet interaction was found at any time point (p>0.05). Overall, the pattern resembled that demonstrated in the chronic study.

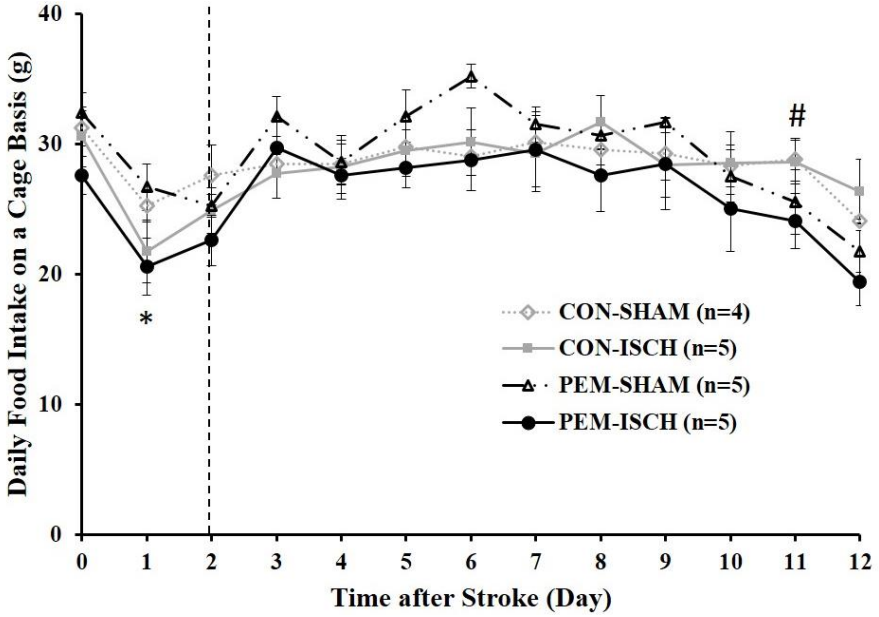

Figure 4.4 Mean (±SEM) daily food intake (g/rat) in the subacute study that estimated based on cage data. Day 0 was the surgery day (the baseline). The dashed vertical line indicates the day on which rats were assigned to receive either control or low protein diet. *indicates significantly lower food intake in pooled ischemic groups compared to combined sham groups (independent t-test, p=0.022). # depicts when the food intake of pooled PEM groups first became significantly lower than that of CON groups (2-factor ANOVA, p=0.025).

4.5 Serum Albumin Concentration

The low protein diet, resulting in PEM, significantly reduced the concentration of serum albumin on both day 12 (2-factorial ANOVA, p=0.001) and day 28 (2-factorial ANOVA, p=0.001) (**Figure 4.5**). Neither a surgery x diet interaction (p_{day12}=0.883, p_{day28}=0.552) nor an independent surgical effect (p_{day12}=0.087, p_{day28}=0.420) was found on either day. On day 12, the serum albumin concentration in malnourished rats was 14.5% lower than that in control animals. With progression of malnutrition to day 28, the serum albumin level of PEM animals was further decreased, resulting in a difference of 22.4% from that of control animals.

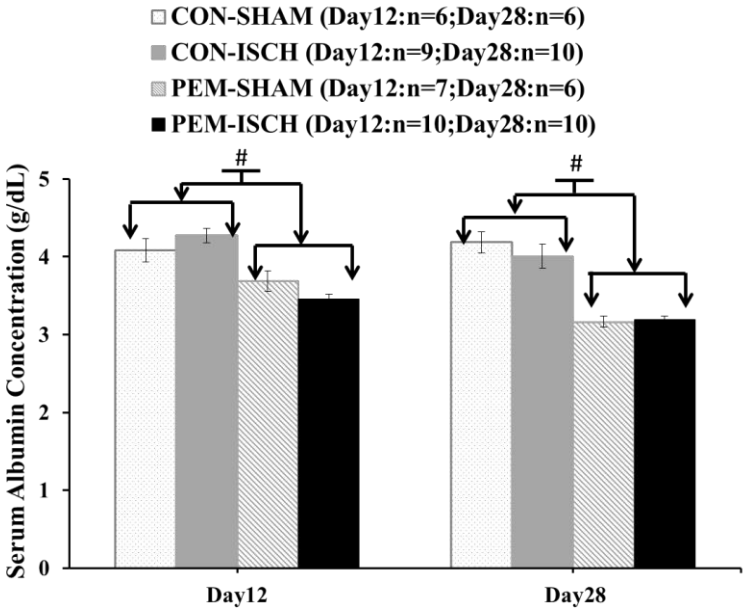

Figure 4.5 Mean (±SEM) serum albumin concentration on days 12 and 28. Animals were assigned to receive either control or low protein diet on day 2 after surgery. # indicates a main effect of diet (2-factorial ANOVA, $p_{day12}<0.001$, $p_{day28}<0.001$).

4.6 Total Liver Lipid Content

The impact of the low protein diet on the total liver lipid content is shown in **Figure 4.6**. On day 12, no significant effect of either diet (p=0.812; 2-factorial ANOVA) or surgery (p=0.624) or their interaction (p=0.586) was found. On day 28, there was a trend for an increase in the liver lipid content in the PEM groups (p=0.051), whereas there was neither a significant surgery effect (p=0.413) nor a diet x surgery interaction (p=0.226).

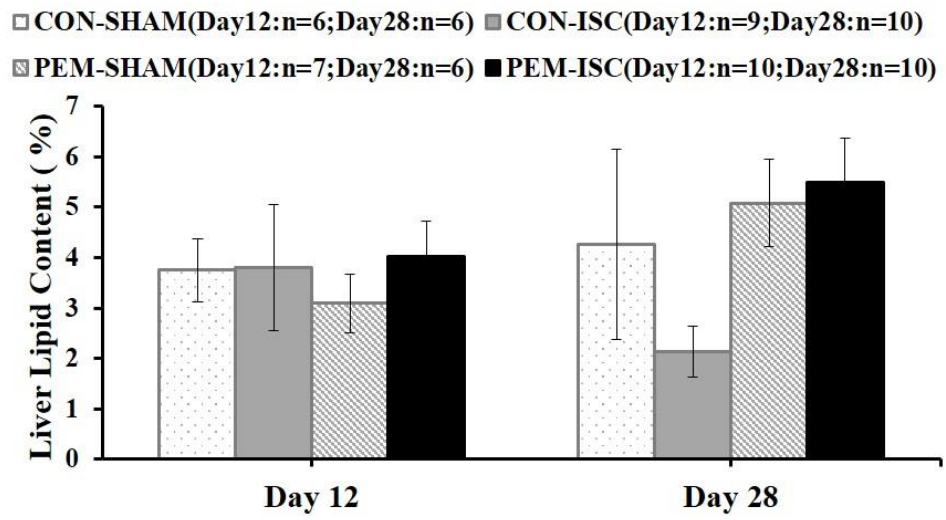

Figure 4.6 Mean (±SEM) total liver lipid content on days 12 and 28. Animals were assigned to receive either control or low-protein diet at day 2 after surgery. There was a trend for the pooled PEM groups to have higher liver lipid content on day 28 (2-factorial ANOVA, p=0.051).

4.7 Functional Assessment: Skilled Walking on the Horizontal Ladder

4.7.1 The Error Rate of the Affected Forelimb on the Regular Rung Pattern

For the chronic study (**Figure 4.7**), 3-factorial repeated ANOVA applied to the error rate of the affected forelimb at baseline and day 2 revealed a significant time x surgery interaction (within-subject effect, p<0.001). The error rate is the sum of rates of slight slip, deep slip and total miss (Metz & Whishaw, 2002). There was no time x diet bias (within-subject effect, p=0.573) or time x diet x surgery interactions (within-subject effect, p=0.692). No difference in performance between pooled ISCH and SHAM groups at pre-stroke baseline was found (independent t-test, p=0.733). The error rate of the affected forelimb of the pooled ISCH groups (32.4 ± 3.1%) was significantly higher than that of the SHAM groups (4.1 ± 1.6%) on day 2 after stroke (independent t-test, p=0.001).

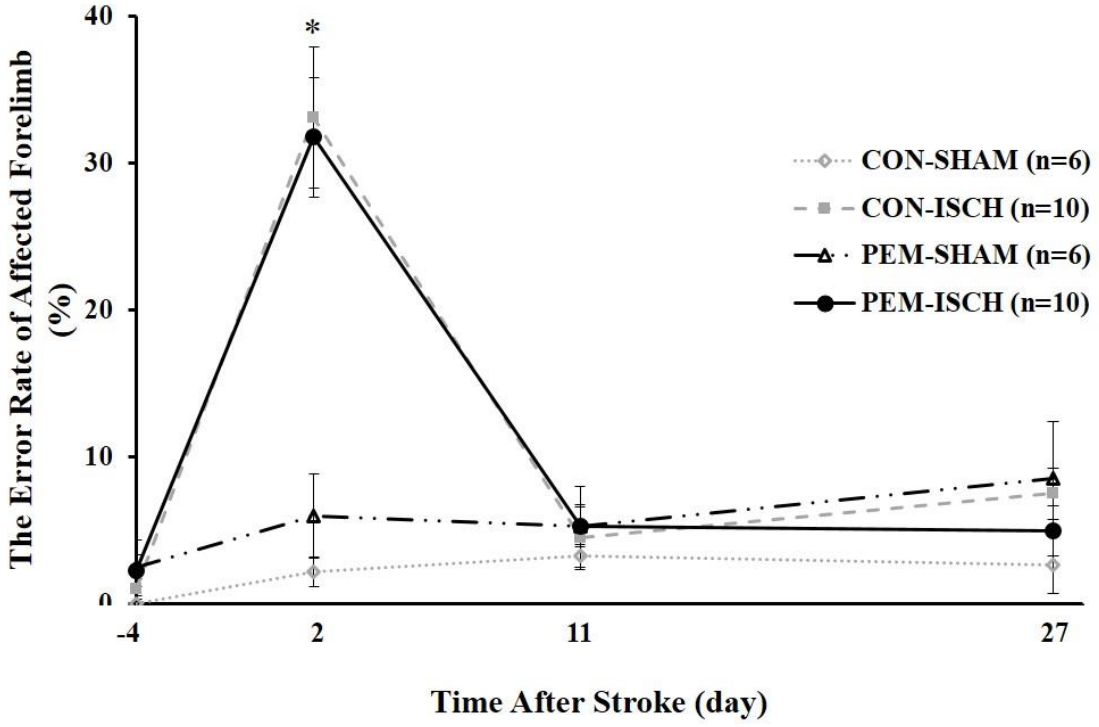

Figure 4.7 Error rate (mean ± SEM) of the affected forelimb on the ladder regular rung pattern in the chronic study. Animals were assigned to receive either control or low-protein diet at day 2 after surgery (before diet assignment). * indicates error rate was increased in ISCH groups on day 2 as determined by 3-factorial repeated ANOVA (within-subject effect, $p<0.001$) and independent t-test ($p=0.001$).

For the chronic study, the analysis of the error rate of the affected forelimb from days 2, 11, and 27 (post-stroke performance) by 3-factorial repeated ANOVA revealed a significant time x surgery interaction (within-subject effect, $p=0.001$) (**Figure 4.7**) that was due to the increased errors in the ISCH groups on day 2. There was no time x diet (within-subject effect, $p=0.558$) or time x diet x surgery interactions (within-subject effect, $p=0.394$). No differences in the error rate between SHAM and ISCH groups were found after day 2 (independent t-test, $p_{day11}=0.758$, $p_{day27}=0.797$).

The error rate on the regular rung pattern in the subacute study was also analyzed to

determine whether changes were consistent with those in the chronic study (**Figure 4.8**). Three-factorial repeated ANOVA analysis of pre-stroke baseline and day 2 data revealed a significant time x surgery effect (within-subject effect, $p<0.001$). An independent t-test found that the error rate of ISCH groups was significantly increased on day 2 ($p<0.001$). In addition, there was a significant time x diet effect (bias) between pre-stroke baseline and day 2 (within-subject effect, $p=0.030$). At the baseline, animals that would be later assigned to CON groups, especially the CON-SHAM group, had a higher error rate than that of animals that would be assigned to the PEM groups (independent t-test, $p=0.034$). Analysis of the error rates on days 2 and 11 by 3-factorial repeated ANOVA showed a significant time x surgery effect (within-subject effect, $p<0.001$). The error rates in the ISCH groups were significantly higher than those of SHAM groups on days 2 and 11 (independent t-test, $p=0.028$). There was no time x diet (within-subject effect, $p=0.174$) or time x diet x surgery interactions (within-subject effect, $p=0.243$). Overall, the trend of changes in the error rate from the subacute study resembled findings in the chronic study.

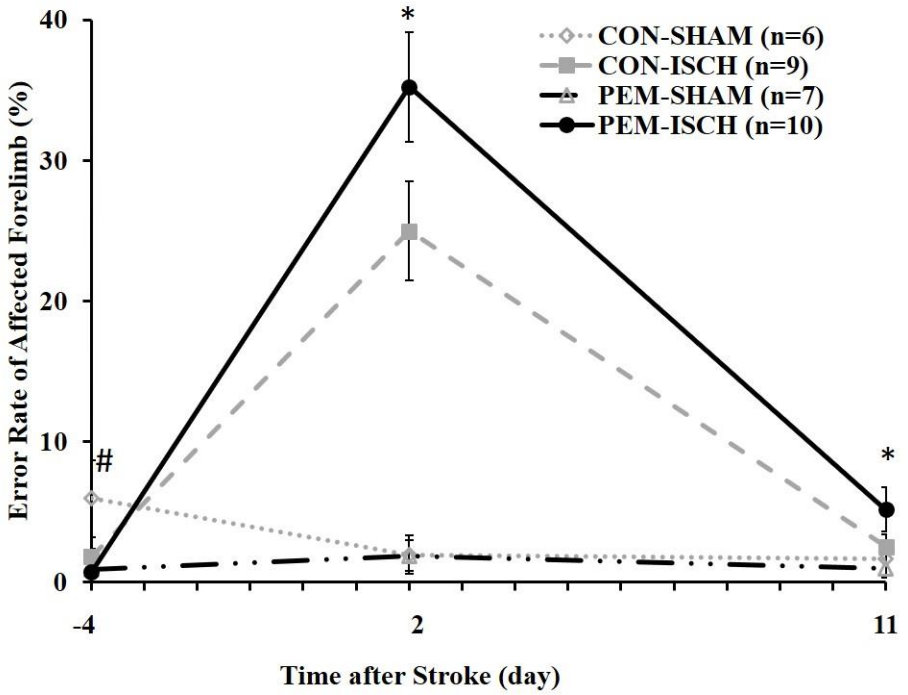

Figure 4.8 Error rate (mean ± SEM) of the affected forelimb on the ladder regular rung pattern in the subacute study. Animals were assigned to receive either control or low-protein diet at day 2 after surgery. *indicates a significantly higher error rate in the ISCH groups (independent t-test, p_{day2}<0.001, p_{day11}=0.028). # significant time x diet effect (pre-diet assignment bias) with rats to be later assigned to CON groups having a higher error rate (3 factor ANOVA repeated measures within-subject effect, p=0.030 and independent t-test, p=0.034).

4.7.2 The Error Rate of the Affected Forelimb on the Irregular Rung Pattern

In the chronic study, the error rate of the affected forelimb on the irregular rung pattern on days 11 and 27 analyzed by 2-factorial ANOVA demonstrated a significant main effect of ischemia on day 11, whereby the ISCH groups had a higher error rate than the SHAM groups on the novel rung pattern (2-factorial ANOVA, p=0.009) (**Figure 4.9A**). There was no independent diet effect (p=0.124), but the diet x surgery interaction showed a trend on day 11 (p=0.075). The higher error rate in the ISCH groups was sustained until day 27, even though the rung pattern was no longer novel (p<0.001) (**Figure 4.9B**). Neither an independent diet effect (p=0.549) nor a diet x surgery interaction (p=0.155) was found on day 27.

In the subacute study, a diet x surgery interaction was found in the error rate of the affected forelimb on the irregular pattern on day 11 (2-factorial ANOVA, p=0.002) (**Figure 4.10**). The error rate of the PEM-ISCH group were significant higher than that of CON-ISCH (p=0.023; Tukey's HSD test) and PEM-SHAM (p=0.006) groups, but not different from that of the CON-SHAM group (p=0.429). There was no difference in the error rate among CON-SHAM, CON-ISCH and PEM-SHAM groups (Tukey's HSD, p>0.05).

Since the irregular rung pattern was tested on day 11 in both chronic and subacute studies with identical study design, the data from the two studies were also pooled to increase the statistical power (**Figure 4.11**). Two-factorial ANOVA analysis of the pooled data revealed a significant surgery x diet interaction (p=0.001), with a significantly higher error rate in the PEM-ISCH group than those of the other three groups (vs CON-SHAM p=0.007, vs PEM-SHAM p<0.001, vs CON-ISCH p=0.001; Tukey's HSD test,). Error rates of CON-SHAM, CON-ISCH, and PEM-SHAM groups were not different from each other (Tukey's HSD, $p\geq$0.553).

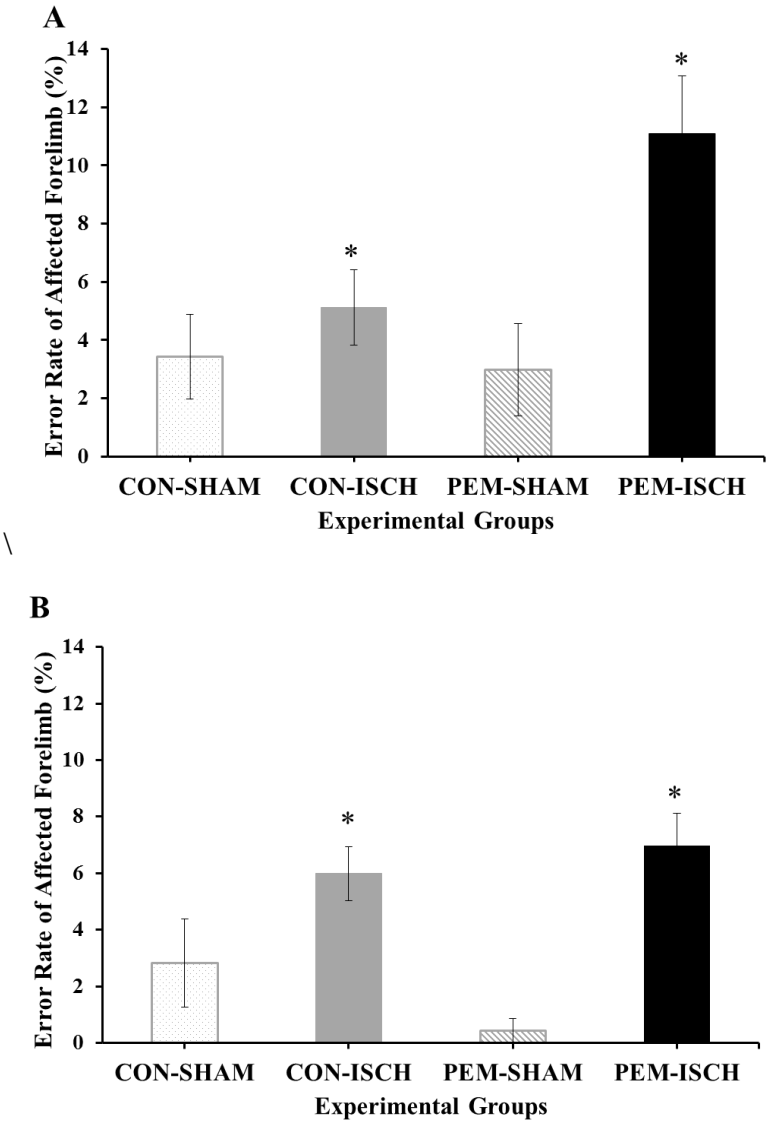

Figure 4.9 Error rates (mean ± SEM) of the affected forelimb on the ladder irregular rung pattern of the chronic study at day 11 (**A**) and day 27 (**B**). * indicates a significantly higher error rate in the ISCH groups, as compared to SHAM groups (2-factorial ANOVA, p_{day11}=0.009, p_{day27}<0.001). CON-SHAM, n=6; CON-ISCH, n=10; PEM-SHAM, n=6; PEM-ISCH, n=10.

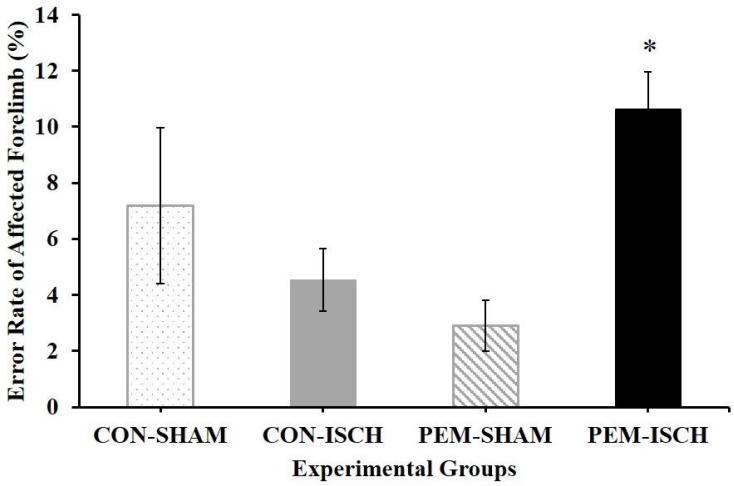

Figure 4.10 Error rate (mean ± SEM) of the affected forelimb on the ladder irregular rung pattern from the subacute study at day 11. * indicates a significant higher error rate in the PEM-ISCH group than that of the CON-ISCH (Tukey's HSD, p=0.023) and PEM-SHAM (Tukey's HSD, p=0.006) groups. The CON-SHAM group was not different from any of the other three groups (p>0.05). CON-SHAM, n=6; CON-ISCH, n=9; PEM-SHAM, n=7; PEM-ISCH, n=10.

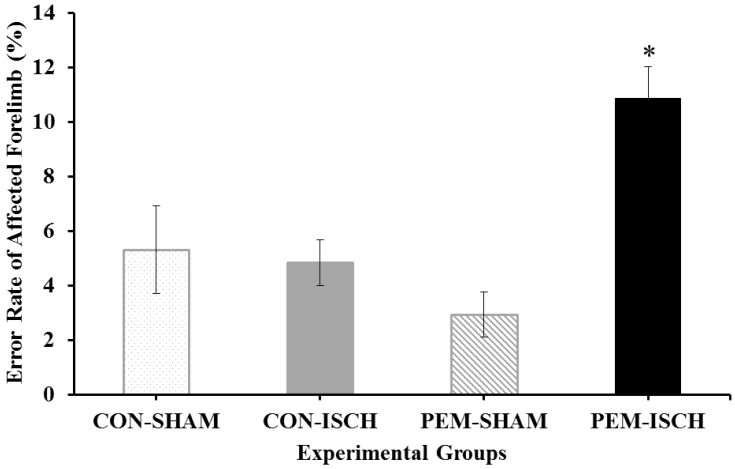

Figure 4.11 Error rate (mean ± SEM) of the affected forelimb on the ladder novel and irregular rung pattern on day 11: analysis of pooled data from the chronic and subacute studies. * indicates a significantly higher error rate in the PEM-ISCH group as compared to all other groups (Tukey's HSD test, to CON-SHAM p=0.007, to PEM-SHAM p<0.001, to CON-ISCH p=0.001). CON-SHAM, n=12; CON-ISCH, n=19; PEM-SHAM, n=13; PEM-ISCH, n=20.

4.8 Infarct Volume Assessment

Representative images of the infarct in CON-ISCH and PEM-ISCH brains observed on days 12 and 28 are shown in **Figure 4.12 A-D**. On day 12, no significant difference between the two groups was revealed for infarct volumes in the cortex (p=0.600; independent t-test), damage in the corpus callosum (p=0.318), or total infarct volume (p=0.528) **(Figure 4.13)**. Tissue loss in the injured hemisphere also did not differ between PEM-ISCH and CON-ISCH groups (p=0.890). Similarly, on day 28, there was no significant difference between experimental groups in cortical infarct volume (p=0.635), corpus callosum damage (p=0.740), total infarct volume (p=0.723), or tissue loss in the injured hemisphere (p=0.814) **(Figure 4.14)**.

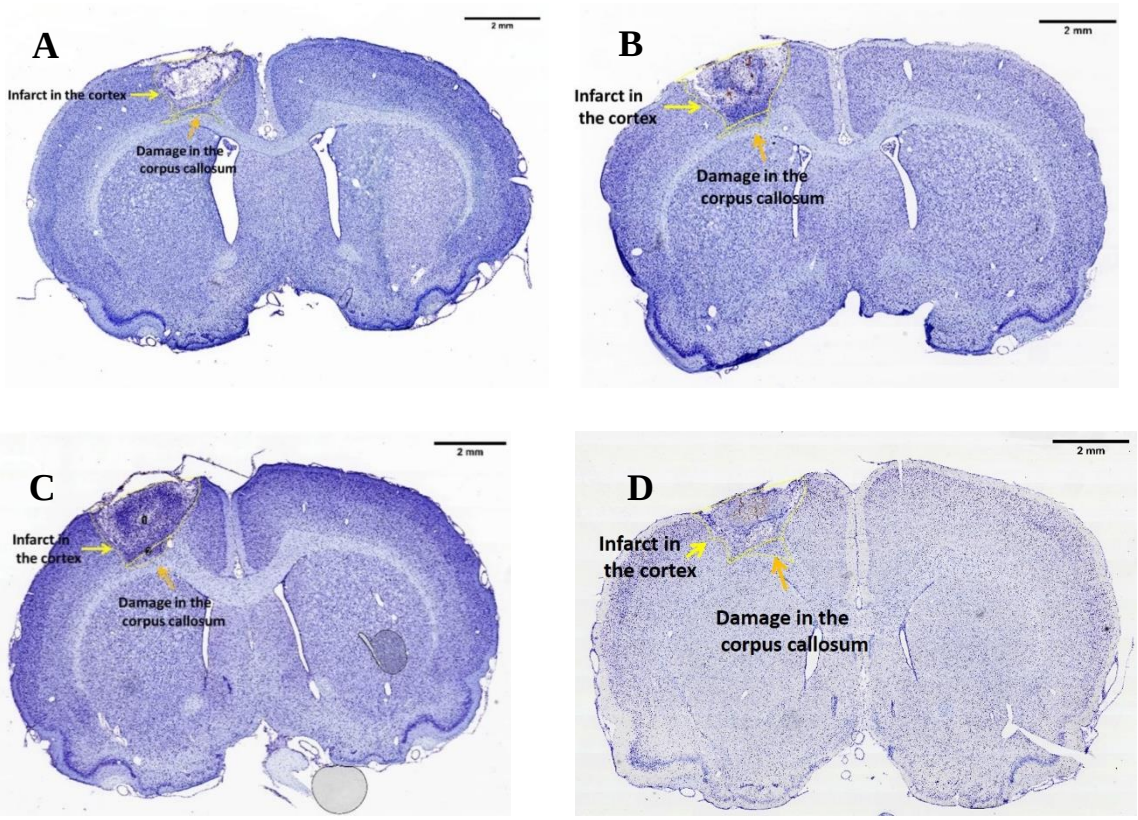

Figure 4.12 Representative cresyl violet stained images of coronal brain sections (28µm thick). A, CON-ISCH brain at post-stroke day 12; B, PEM-ISCH brain at post-stroke day 12; C, CON-ISCH brain at post-stroke day 28; D, PEM-ISCH brain at post-stroke day 28. Varied colour intensity in each image is due to different batches of staining (See Appendix A).

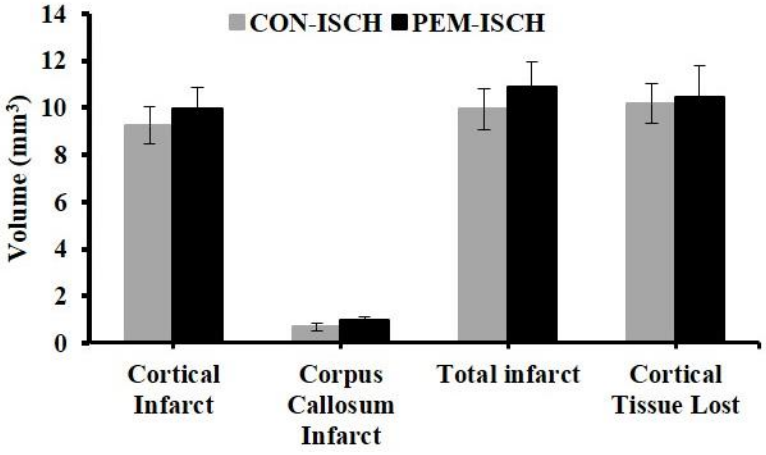

Figure 4.13 Infarct volumes (mean ± SEM) on post-stroke day 12. Animals were assigned to receive either control diet (CON-ISCH) or low protein diet (PEM-ISCH) on day 2 after surgery. There were no significant differences between CON-ISCH and PEM-ISCH groups for any endpoint (independent t-test, $p>0.05$). CON-ISCH, n=9; PEM-ISCH, n=10.

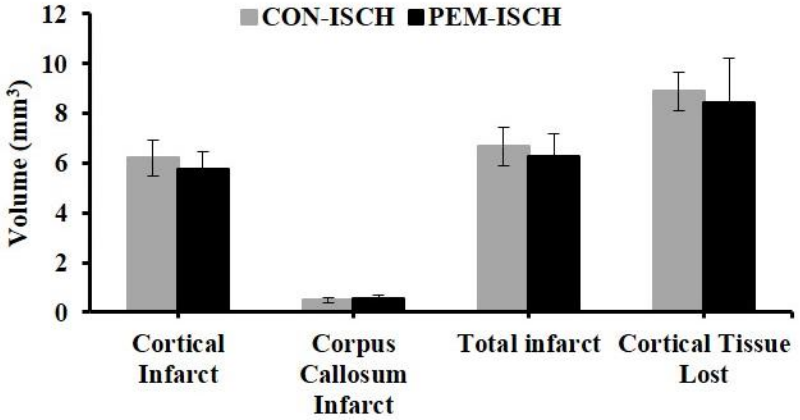

Figure 4.14 Infarct volumes (mean ± SEM) on post-stroke day 28. Animals were assigned to receive either control or low protein diet on day 2 after surgery. There were no significant differences between CON-ISCH and PEM-ISCH groups for any endpoint (independent t-test, $p>0.05$). CON-ISCH, n=10; PEM-ISCH, n=10.

Chapter 5 DISCUSSION

Well-characterized rat models of PEM (Alaverdashvili *et al.*, 2015a) and cortical stroke (Alaverdashvili *et al.*, 2015b) were used to test the hypothesis that protein-energy malnutrition developing after stroke would impair functional recovery. The major finding is that post-stroke PEM induced by a low-protein diet worsened the recovery in walking ability without altering the infarct size. However, this effect of PEM was evident only under the most challenging condition. The finding partially supports the hypothesis and demonstrates that post-stroke PEM can be detrimental to stroke recovery. Therefore, clinical measures should be taken to prevent and treat this common co-morbidity.

Post-stroke protein-energy malnutrition was successfully induced by feeding a low protein (0.5%) diet to adult male rats beginning on day 2 after stroke. Evidence for PEM was provided by the decrease in body weight, food intake, and serum albumin concentration, which is consistent with our previous results (Andrade Ramos, 2013; Alaverdashvili *et al.*, 2015a; Matwee, 2016). There was also a strong trend for increased total liver lipid content in low dietary protein-fed groups, which was only evident by day 28. This provides further evidence of protein-energy malnutrition. Comparing with our previous studies where a significantly increased total liver lipid was typically found in malnourished rats (Andrade Ramos, 2013; Alaverdashvili *et al.*, 2015a; Matwee, 2016), the large variability seen in my CON-SHAM group likely influenced the statistical result. Although there was an initial bias in body weight despite random group assignment, the pattern of changes in body weight and food intake induced by the low dietary protein in the subacute study resembled that of the first 12 days of data from the chronic study. The decline in serum albumin concentration was seen on day 12. In fact, this decrease occurs as early as 3 days after introducing a low protein diet (Alaverdashvili *et al.*, 2015a). It is important to mention that serum albumin concentration is not a specific marker of PEM; being a negative acute phase protein, it can be affected by both dietary amino acid supply and an acute phase response (Qu *et al.*, 1996). Thus, other nutritional indices, such as food intake, body weight, and liver lipid, were important to confirm the nutritional status.

The stroke model introduced some challenges in testing the hypothesis that post-stroke PEM would delay or reduce functional recovery. The increased error rate on the horizontal ladder at post-stroke day 2 in two ischemic groups demonstrated that a deficit in skilled walking was induced by stroke (**Figure 4.7**). However, when tested on the regular rung pattern again on post-stroke days 11 and 27, malnourished and control rats subjected to stroke from both studies showed almost complete recovery, with their function resembling that observed in sham groups (**Figure 4.7**). This demonstrated that the permanent functional deficit aimed for was not achieved. An ideal recovery profile after stroke in a preclinical animal model should be incomplete, mirroring that of many stroke patients (Krueger *et al.*, 2015; Corbett *et al.*, 2017). Besides not providing a good mimic of a stroke patient, this limitation reduced the sensitivity to detect any potential effects of PEM, since recovery occurred before PEM could fully develop. The small but statistically significant differences among groups observed in skilled walking data between subacute and chronic studies also demonstrate the high variability that is common to behavioral studies and emphasize the need to use large sample sizes for behavioral endpoints. In these two studies, a total of 22 sham rats had to be excluded due to the unintentional cortical damage caused by excessive force applied during skull drilling during surgeries; this resulted in the small sample size of the sham groups.

Increasing the walking challenge in the ladder did reveal a more persistent walking deficit after stroke. When challenged with a novel irregular ladder rung pattern on day 11 after stroke, and the same irregular pattern on day 27, a higher error rate was found in well-nourished (control) rats exposed to stroke, compared to sham groups. Thus, CON-ISCH animals had not completely recovered from their stroke deficit. The irregular rung pattern, which was more challenging and novel (but only on day 11), mimics the "real life" environment with its variety of walking challenges and unpredictable obstacles that require adaptations.

The added sensitivity of the irregular rung pattern also discerned that PEM exerted some detrimental effects on post-stroke walking recovery that was dependent on the extent of the walking challenge presented. The error rate on the irregular pattern in the PEM-ISCH group was

significantly higher than that of each of the other three groups on day 11 when data of subacute and chronic studies were pooled together to increase the power (**Figure 4.11**). This indicates that protein-energy malnutrition decelerated the rate of walking recovery. Note, however, that this was not a consistent finding when the data from subacute and chronic studies were individually analyzed. On post-stroke day 27, when the irregular rung pattern was no longer novel, the PEM-ISCH group performed similarly to the CON-ISCH group (**Figure 4.9B**). This might indicate that PEM impairs the ability to overcome novel walking obstacles efficiently. Testing of this hypothesis would require further studies with repeatedly novel rung patterns, versus familiar ones, presented at several different times after stroke. Nevertheless, my results suggest the importance of treating post-stroke protein-energy malnutrition to achieve optimal recovery after stroke.

Detrimental effects of PEM on recovery of walking ability are not explained by an increase in infarct sizes. Using two methods to evaluate the volume of brain damage, there was no difference in infarct sizes between PEM-ISCH and CON-ISCH animals on either day 12 or day 28 (**Figures 4.13 and 4.14**). Although it is ideal to include the entire hemisphere in such assessments (Clark *et al.*, 2008), only the cortex in each hemisphere was examined due to staining and sectioning artifacts that damaged subcortical structures and precluded the possibility of outlining the entire hemisphere. Overall, this finding is in line with our previous observations with both PEM pre-existing at stroke onset (Alaverdashvili *et al.*, 2018) and PEM developing after stroke (Matwee, 2016). Since the experimental diet was not started until post-stroke day 2, it was not expected that PEM would influence the infarct size as the infarct core should have been well developed by then (Lee *et al.*, 1996). Thus, effects of PEM on post-stroke recovery of walking are exerted through other mechanisms.

There were no differences between the two ischemic surgical groups (later assigned to PEM or CON diet) in the physiological parameters measured during the surgery. Thus, there was no confounding influence of these variables on study results. Mean body temperatures of ischemic groups were higher than those of sham groups by 0.5 degree in both subacute and chronic studies. It has been previously reported that certain stroke models can induce changes in the body

temperature while others do not (Klahr *et al.*, 2017). Although hyperthermia is known to increase infarct volume, such an effect typically requires a dramatic increase (2-3 degrees) (Reglodi *et al.*, 2000; Meng *et al.*, 2012).

There are several mechanisms by which post-stroke PEM could worsen recovery of walking ability. First, our previous results demonstrated that PEM alone (without stroke) can induce a gait abnormality in the horizontal ladder walking task in adult rats, which can be partially explained by selective skeletal muscle atrophy in hindlimbs (Alaverdashvili *et al.*, 2015a). However, this did not cause an increase in the error rate on the horizontal ladder, such as was observed after stroke. Secondly, while PEM pre-existing at stroke onset can attenuate microglia and astrocyte activation in the peri-infarct area at 3 days after stroke (Alaverdashvili *et al.*, 2018), it is possible that PEM developing after stroke could also influence the stroke-induced glial response. Thirdly, I examined expression in the peri-infarct region of two axon terminal proteins that serve as markers for synaptic remodeling, GAP-43 (Carmichael *et al.*, 2005) and synaptophysin (Stroemer *et al.*, 1995), in my related nonthesis research,. No significant differences were found between PEM-ISCH and CON-ISCH animals on either day 12 or day 28 (unpublished data), suggesting that PEM may not influence walking recovery through the modification of neuroplasticity in the peri-infarct region. However, the immunohistochemical approach used was limited in power, being semi-quantitative in nature, and we examined only two neuroplasticity-related proteins. A more comprehensive analysis would be required to exclude this mechanism.

Canadian Stroke Best Practice Guidelines recommend that nutritional status in stroke patients should be screened within 48 hours of admission and those with suspected malnutrition should be referred to clinical dietitians for detailed assessments (Casaubon *et al.*, 2016). Rescreening for changes in nutritional status is also recommended throughout the hospital stay and prior to discharge (Hebert *et al.*, 2016). Periodically, rescreening should be carried out in rehabilitation and community settings to facilitate creating the optimal management plan for individual patients (Hebert *et al.*, 2016). Findings from this thesis project support these national guidelines and emphasize potential adverse functional consequences of not treating post-stroke protein-energy malnutrition.

The study had several strengths and limitations. By using well established protein-energy malnutrition (Alaverdashvili *et al.*, 2015a) and photothrombotic stroke (Alaverdashvili *et al.*, 2015b) models, it was possible to study the effect of post-stroke PEM on stroke recovery without confounding influences inherent in complex patients in clinical studies. This allows a cause and effect relationship to be studied. Another strength of the study was the inclusion of a functional outcome, the major end point in clinical studies (Stroke Therapy Academic Industry, 1999), at acute, subacute and chronic post-stroke stages, in order to study effects on the progress of stroke recovery. Including a familiar regular rung pattern and an irregular pattern (with and without novelty) provided different degrees of walking challenge and increased the sensitivity of the task for testing the effects of PEM. As noted above, the primary study limitation was that a more severe and lasting functional deficit was not achieved with the stroke model. This reduced the sensitivity to detect any potential effects of the PEM, since recovery on the easy walking paradigm occurred before PEM could completely develop. Another limitation discussed above was the unfortunate loss of a significant number of sham-operated rats, which resulted in unequal sample sizes across experimental groups. A more complete understanding of the effects of PEM on motor recovery after stroke would have been obtained by employing a battery of motor behavior tasks that are validated for cortical stroke models. Such tests include the Montoya staircase (Montoya *et al.*, 1991), cylinder task (Schallert, 2006), tapered beam walking task (Goldstein & Davis, 1990; Schallert *et al.*, 2002; Zhao *et al.*, 2005), and single pellet reaching (Whishaw *et al.*, 1991). Unfortunately, our laboratory has shown that tests that require extensive food rewards for training, such as the Montoya staircase that assesses skilled reaching, are not valid for studies of PEM as it was found in our lab that the motivation for obtaining sugar pellets was significantly elevated in malnourished rats (unpublished data). Therefore, one might only be assessing motivation instead of assessing motor function. The cylinder task has been utilized in our previous studies, and it was shown that post-stroke PEM prevented recovery of use of the affected forelimb during spontaneous exploration (Matwee, 2016). In future, the tapered beam walking task would be a useful addition to the horizontal ladder to investigate the effect of PEM on post-stroke skilled walking. Rats make

fewer compensatory adjustments in this task, allowing one to measure a deficit unconfounded by compensation (Schallert *et al.*, 2002).

The study results leave many unanswered questions that deserve further investigation. Different infarct sizes could be used to study effects of untreated PEM on varying degrees of stroke-induced walking deficits. Using repeatedly novel irregular rung patterns on the ladder at several post-stroke times could be used to test the possibility that PEM impairs the ability to overcome novel walking obstacles after stroke. Underlying mechanisms through which PEM interferes with walking recovery after stroke also require further investigation. As elderly individuals are most vulnerable to stroke (Livingston-Thomas *et al.*, 2016), an important future experiment would be to investigate the effects of post-stroke PEM in aged animals.

In addition, a growing body of evidence has demonstrated secondary damage in brain regions distal to a primary cortical infarct (e.g. thalamus and hippocampus) in both human patients and animal models (Zhang *et al.*, 2012; Carrera & Tononi, 2014). These secondary brain injuries have been associated with both motor and cognitive deficits (Tamura *et al.*, 1991; Zhang *et al.*, 2012; Buma *et al.*, 2013; Carrera & Tononi, 2014). Such secondary injuries have not been studied in a photothrombotic stroke model, which would be interesting to investigate in the future. Since PEM (without stroke) can cause morphological (Andrade *et al.*, 1996; Lukoyanov & Andrade, 2000) and functional abnormalities in the hippocampus (Nakagawasai *et al.*, 2006; Wang & Xu, 2007; Soares *et al.*, 2013; Belluscio *et al.*, 2016; Perez-Garcia *et al.*, 2016), it would be of interest to investigate whether PEM also influences post-stroke recovery by either aggravating secondary hippocampal damage or through independent effects on the hippocampus.

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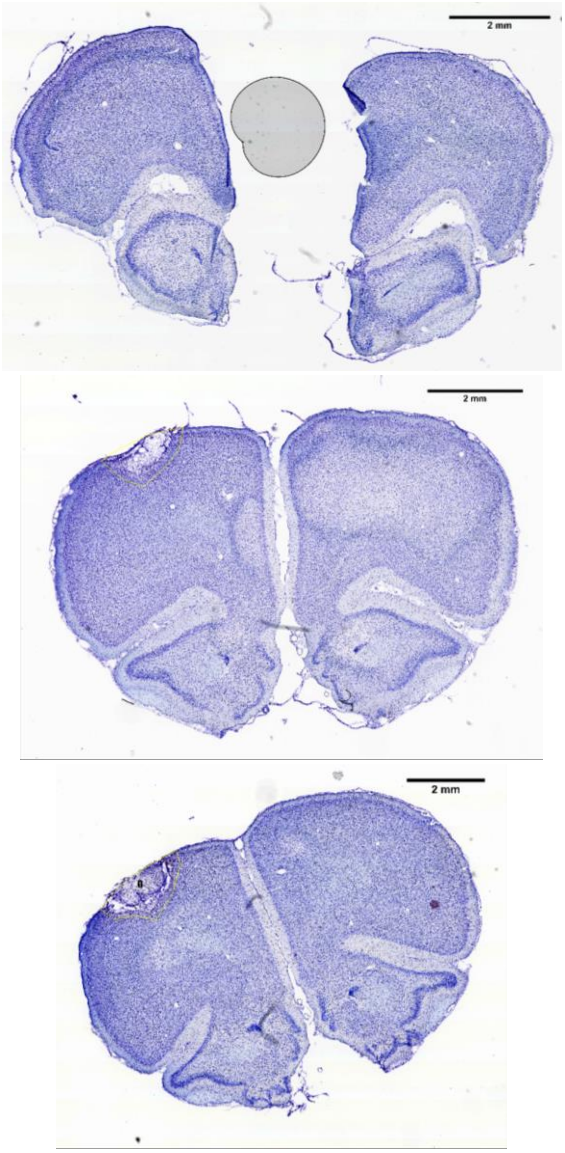
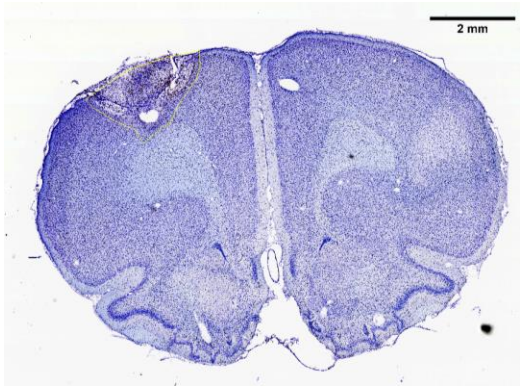
APPENDIX A

This set of images of coronal sections stained with cresyl violet from a post-stroke D28 CON-ISCH brain demonstrates a representative series of images used to evaluate infarct volume. Each section is 28 µm thick and the sections are 420 µm apart. The yellow tracing separates the outline of cortical damage from the damage to the corpus callosum (the first method in section **3.9**).

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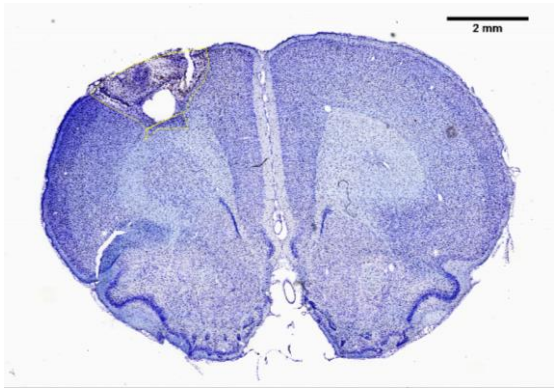
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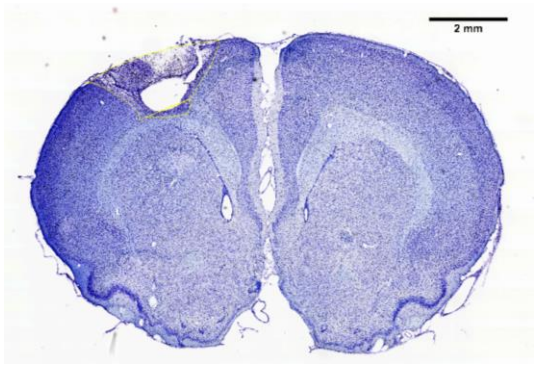
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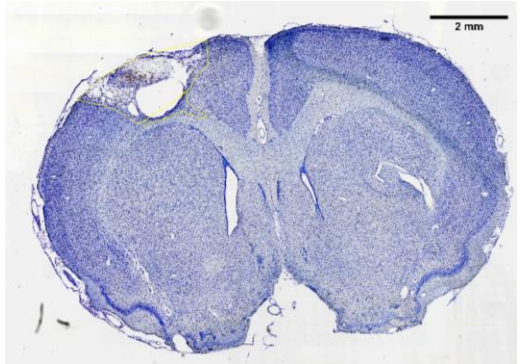
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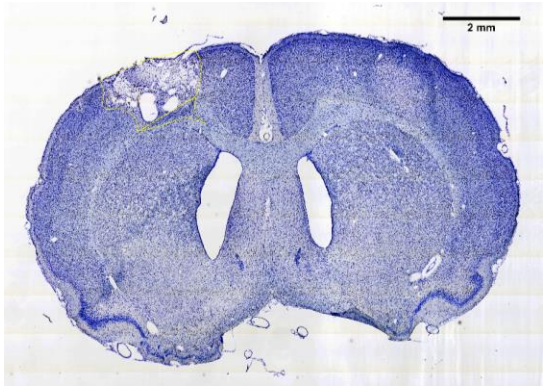
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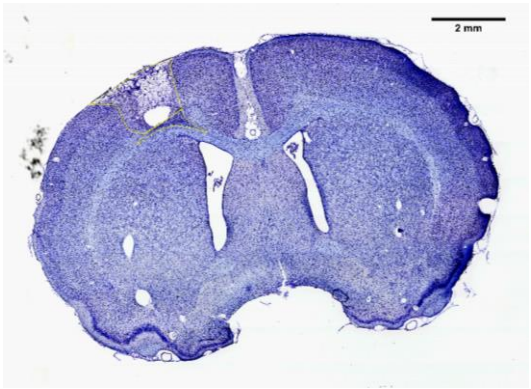
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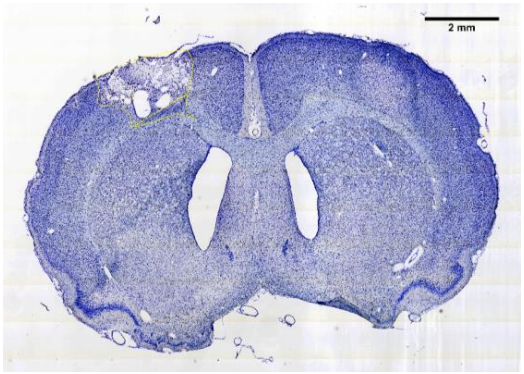
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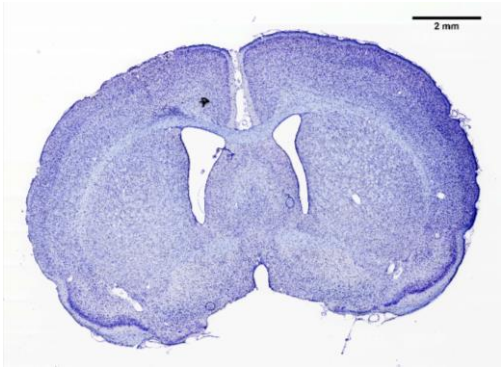
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